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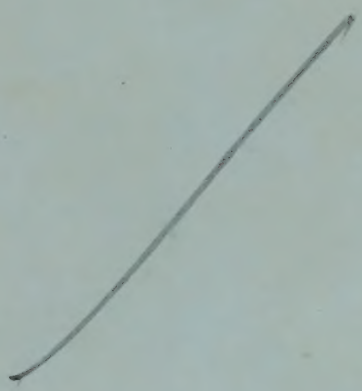

STARCH

AND ITS DERIVATIVES

By

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Chemical Consultant

BEING VOLUME ELEVEN OF A SERIES OF MONOGRAPHS ON APPLIED CHEMISTRY

Under the Editorship of
E. HOWARD TRIPP, Ph.D.

THIRD EDITION
(REVISED)

VOLUME ONE

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE,
LIBRARY
-7. AUG. 1954
* MYSORE. *

LONDON
CHAPMAN & HALL, LTD.
37 ESSEX STREET, W.C.2
1953

First published 1940

Second edition revised . . . 1943

Third edition (in two volumes) . 1953

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E1, 58768

N53.1

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CATALOGUE NO. 160/4

PRINTED IN GREAT BRITAIN BY THE ALCUIN PRESS LTD., WELWYN GARDEN HERTS.

BOUND BY G. & J. KITCAT LIMITED, LONDON

EDITORIAL PREFACE

ese days of intensive and extensive research, every worker ence or its applications knows how rapidly the contents of ooks and encyclopædias become out of date; and those vish to see new work published know the difficulties which mal taxation and high labour costs offer to the realisation of desire. The one obvious solution of the problem is the cation of monographs that would focus attention upon t work, or upon new aspects of old work, and upon their etical implications. Such books are usually written by ts for other experts in related fields of science, or for the educated layman whose thirst for new knowledge has not been ched by the more sensuous outpourings of the ephemeral press. s interesting at times to speculate upon what aspects of our ation the future historian will select as the most character- of our time. Scientific discoveries and their application to n welfare, we may be sure, will find a place; and to these y will add the growth of our sense of 'values'. The value of work in science varies greatly: the golden grain is always ompanied by chaff, and there is no precious ore without ntry rock. Owing to the difficulty of assessing the value of rk at the time of its production, we find that our scientific riodicals stand in danger of being swamped by the mass of cond- and third-rate material that is thought to be worth blishing, but which posterity will decree would have been tter left in manuscript form. It is the first duty of the mono- aph writer to estimate the value, either actual or potential, of cent work upon the subject of which he writes: he must pick t the plums to save others from the indigestion that follows ing the whole pie. Further, in addition to being accurate, his rk must be presented in a form that is both assimilable and tractive; in other words, he must show that lucid exposition an be achieved by the use of few words, if they are rightly chosen, and that attractive presentation is attained rather by clear thinking than by superficial display.

The present series of monographs has been designed with these objects and ideals in view. The task which the authors have been set is no easy one; so that should performance occasionally fall short of intention, the critical reader is asked to echo the ds of Goethe that 'higher aims, even if unfulfilled, are in nselves more valuable than lower aims quite attained'.

E. HOWARD TRIPP.

AUTHOR'S PREFACE

It is hoped that this book will recommend itself to many sc and technical workers without any lengthy apologia fro author. Like all the books that have appeared in this serie one seeks to summarise recent progress in an important c of chemical industry ; for starch, if not the food of the goc fundamental necessity to all human beings. Though much text is descriptive, critical commentary has not been forg The author hopes that his own experience of the indust helped him to preserve a balance between the purely sci and the technological, and to give just weight to diverse vie controversial points. As the manufacture of the various mercial kinds of starch has already been described at len several useful books, this part of the subject has been treate broad, general way ; *per contra*, the manufacture of de about which little has appeared in print, has been treated in Notwithstanding the very great amount of work already do starch and its derivatives (as the numerous references t literature testify), the careful reader will not fail to discern a gap in our knowledge ; and it is hoped that he, whether h purely academic or a technical worker, will find in these some food for thought and some material upon which to further investigations.

I have pleasure in acknowledging the help I have received firms and individuals in compiling this volume. My thank due to Messrs. Imperial Chemical Industries, Ltd., for generous permission to use their photomicrographical appar and to Mr. E. Young for placing at my disposal his great m graphic skill ; to Drs. S. H. Oakshott, R. J. W. Reynolds, Me H. Blackshaw, J. Faulds, P. T. Gale, N. Strafford, and J Kierman for their valuable criticisms ; to the editors of va publications and to firms who have lent blocks for diagrams apparatus ; to Prof. D. H. Cook for providing samples of tro starches, and to other workers who have sent samples of star or reprints of their publications. Finally, but by no means l I thank the editor of the series and the publishers for t valuable work in converting the original manuscript into finished book.

J. A. RADLEY.

THOR'S PREFACE TO SECOND EDITION

need to commence an edition of this book only some eight hs after the appearance on the market of the first edition oof of the sustained interest in the subject of starch in all rious aspects. It is all the more interesting in view of the ations of circulation due to the present state of the European Asiatic starch-producing countries.

this present edition of thirty-four chapters and an appendix h new material has been included, comprising some eleven ely new or completely re-written chapters and about 1,200 references. The chapter on Physical Chemistry has been vided into some five shorter chapters as, with the addition e new material, it would have been decidedly unwieldy. new arrangement, it is hoped, will allow of easier reference. new photographs and diagrams have been added better strate both the previous matter and the new material.

ny new papers on starch and its uses are constantly coming rd and many of the older observations have received fresh icance and explanation. A certain amount of the old work een discarded although in many cases the references to it in for those who wish to follow the subject through every of its development. The uncertain or non-arrival of papers Germany and the occupied countries, and the pressure of circumstances at every stage have all contributed to the lties of production of this edition, but it is hoped that it les all the more important work published to the end of

J. A. RADLEY.

AUTHOR'S PREFACE TO THIRD EDIT

THE present edition of this work was called for some nine mo
after the appearance of the second edition in 1943. Despit
non-availability of recent literature from Europe, the unce
and irregular arrival of American journals and the drain im
upon mental energy bent towards problems of 'national im
ance' during practically the whole of the waking hours i
decided to commence a third edition in the hope that the
would be nearly ready for press a short time after the cess
of hostilities and that too many papers would not appear
abroad to invalidate the partial labour already accompli
Further it was anticipated that the paper position and produ
situation would ease to some appreciable degree with the e
the war.

Events have demonstrated the falsity of all these hopes.
last eight years have seen remarkable achievements in the el
tion of the problems of starch structure, chemistry and beha
These have necessitated considerable re-writing of large po
of the book if the aim of presenting a complete and up-to-
picture of the present state of our knowledge on the subject, w
to be preserved. In practice this aim can only be striven for an
never achieved unless the subject reaches a static conditio
Post-war paper and production problems have widened the g
between the goal and its achievement since, despite all th t t
publishers and the author can contrive, the time between t
submission of the MS. and the appearance of a book is very mu
wider than is desirable or than it used to be pre-war. Th
portions of a book are liable to be out-dated whilst in the p
proof or binding stages.

The immensity of the subject determined the writer to exte
the precedent, set in the Second Edition, of inviting the assistan
of prominent workers in specialised fields of starch technolo
and chemistry in the task of presenting specialised sections i
concise and authoritative manner. This has led to the welco
sta lition of more American contributions which, it is hoped,
or re a better representation of American work, practice
I thar s.
valuabl he contributors have generously given me most valu
finished e, some in the face of overwork and ill health, and
me, the disappointments attendant on the variou
delays that have occurred.
d, therefore, that the present edition is as up to
the accumulated knowledge on the chemistr

ology of starch and its derivatives as possible, but possibly papers of importance from abroad may have been omitted o their arriving too late for inclusion.

e general method of presentation used in the earlier editions ed general appreciation and has, therefore, been resumed e present edition. A number of new chapters have been , and many of the older ones have been completely re- n and extended by authorities in the particular fields with the chapters deal. The chapter on the waxy cereals and es is, it is believed, the first comprehensive account of the y, genetics and agronomy as well as the chemistry of these ials that has appeared in print and it is hoped it will be of l interest to those whose work lies in these domains. There some original work included, notably in the contribution . Thomas Schoch.

there is much of interest in the older work, providing the l side only of this work is admitted, many of the references been retained, generally in the form of 'Additional Refer- '.

the preface to the Second Edition reference was made to mber of people who helped then and it is with pleasure that writer again acknowledges the valued help of most of them. writer's thanks are renewed to G. V. Caesar for the additional ter on 'Dextrinisation' and Dr. R. W. Kerr and Dr. T. J. ch deserve particular thanks not only for their contri- ns but the very personal interest they have taken in the through all its stages. It gives me pleasure also to extend hanks to Dr. S. Peat for his assistance which was ren- in circumstances of overwork and ill health which would quite justified his withdrawing from the venture had he so minded. Thanks are due, also, to Dr. J. K. N. Jones oroughly revising the chapter on 'The Structure of Starch Chemical Evidence', written originally by Professor E. L. nd Dr. Young for the Second Edition; to Dr. Roy Whistler; fessor R. M. Hixon and Miss Bernadine Brimhall for their e contribution on the waxy cereals and starches, alre ded to a ned above; to Professor Ed. F. Degering, who took stained blue-v of no less than four chapters on his shoulders; to Letely destroyed nsen and L. A. Underkofler for their chapter peared to distingui ion of Chemicals from Starch by Fermentation Pect was taken up in C. W. Bice and Dr. W. F. Geddes for their 28 rather discursive p tion on the role of starch in bread stalhease was a new enzyme no means least, to C. C. Kessler and W It was separable from an ntribution on 'The Physical Methods by precipitation with lead ac

Nishim
an enzym

Starch', which will undoubtedly be found extremely valua b control chemicals and research workers alike. Some portis the book appear at first sight to overlap small sections thi last contribution, but closer examination will reveal that, imes cases, the information is really complementary, so that neon densation has been made which would have tended to intrup the development of the presentation in the few places her overlapping has apparently taken place.

A number of workers continue to send me reprints ofhei papers on starch and, some, letters of constructive criticism T all of these I tender my thanks for their kindness, and hop th practice will continue.

Finally, I should like to express my appreciation of thgrea help and encouragement I have received from the publishe an their staff who have laboured to translate this work from m t print, sometimes under the most frustrating circumstances

Over the space of five years the difficulties of writing, colbor ating with distant workers and producing a book covering va and rapidly expanding subject always introduces the possilit of omissions, errors, repetition and even minor contradictio but in this matter the writer asks the reader for some indulger and expressed the hope that something of merit and of value m be found in this volume. If this hope is realised then the arduo task has not been attempted in vain.

J. A. RADLEY.

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the reaction and is the sole product of the hydrolysis of unbranched amylose structures. Amylopectin also yields maltose under the action of β-amylase, but the branch points in this starch component obstruct the action of the enzyme and, in addition to maltose, limit dextrins result which do not function as substrates for further β-amylolysis. It is a striking fact that whereas the sugar produced by amylolysis *in vitro* is maltose, this disaccharide has never been detected in more than traces in any plant tissue. This apparent anomaly may be ascribed to the presence in most tissues of a maltose-splitting enzyme which converts the maltose as it is formed into glucose. But this is not the only anomaly. It is not possible to convert the amylopectin component of starch completely into glucose by any laboratory combination of α- and β-amylase and maltase. It may be that the removal of the enzymes from their cellular environment and the resulting disorganisation of the whole amylolytic system destroys some vital element which in the living cell enables the complete conversion of starch to glucose to take place. Yet it must be remembered that there is no direct proof that the starch which disappears during the course of the metabolic cycle, is in fact converted into glucose. It is true that the glucose content increases as the starch decreases, but so do the fructose and sucrose concentrations and there are certain indications from recent work[10] that the starch/sucrose balance in plants may be of more significance than the starch/glucose balance.

Synthesis.—Until very recently nothing was known of the mode of biogenesis of starch and it was reasonably assumed, in default of evidence to the contrary, that synthesis was merely a reversed amylolysis. Nevertheless all attempts to detect a synthesising function in the known amylases have failed completely. The enzymic conversions of starch into maltose, and of maltose into glucose are irreversible reactions *in vitro*. Claims have been made, however, for the existence of enzymes which catalyse the conversion of short chain dextrins into starch. In 1930, Nishimura reported[11] the extraction from yeast autolysates of an enzyme—the so-called amylosynthease—which when added to a non-staining achrodextrin gave a product which stained blue-violet with iodine. The amylosynthease was completely destroyed by a temperature of 55-60° C., a fact which appeared to distinguish it from either α- or β-amylase. The subject was taken up in 1932 by T. Minagawa who in a series of 28 rather discursive papers produced evidence that amylosynthease was a new enzyme independent of the known amylases.[12] It was separable from amylase by extraction with glycerol and by precipitation with lead acetate,

ammonium sulphate or cadmium chloride. Later Minagawa came to the conclusion that both amylase and amylosynthease exist in the plant as zymogens and that it was possible to separate the zymogens by extraction of the amylase-zymogen with aqueous solutions of various inorganic salts. When amylase and amylosynthease were mixed an antagonistic action was observed, as was demonstrated by the much longer time required for synthesis if the amylosynthease contained amylase.

Amylosynthease is widely distributed in the plant kingdom. It has been isolated from the potato and other tubers, from the seeds of rice, glutinous rice, maize, wheat, millet, buckwheat, rye and panic grass. Polished rice is especially rich in amylosynthease.

This work is open to one serious criticism. No attempt is made to isolate and examine the product synthesised from dextrins and the sole criterion of synthetic action is the genesis of a substance which gives a blue-violet colour with iodine. Even this development of iodine-staining power is not examined quantitatively and the control experiments are inadequate. Nevertheless, one feels that here is something new in the amylase field, something that would well repay closer investigation by the refined methods which have been developed during the past decade. The writer has been impressed by the close similarity in properties between amylosynthease, reported by Minagawa, and the Q-enzyme, isolated from the potato, which is involved in the synthesis of amylopectin.[13, 14]

Phosphorolysis and Phosphogenesis.—The conception of starch synthesis as a reversed amylolysis lost favour when positive evidence began to accumulate of the existence of a very different biological mechanism of synthesis. The evidence came, in the first place, from the parallel studies of the synthesis of glycogen in the animal and was in the main contributed by the brilliant work of C. F. Cori and his school. It was shown that the intermediate in the breaking down and building up of glycogen in muscle was neither free glucose nor maltose but rather was glucose phosphorylated in position 1 (the Cori ester).[15] Furthermore, the phosphorylase catalysing this interconversion was isolated from a number of animal tissues and that from muscle was ultimately obtained in the crystalline state.[16]

In 1940, Hanes[17] demonstrated the presence of a phosphorylase system also in the higher plants and was able to isolate from the pea and the potato an enzyme which catalysed the reversible reaction.

Glucose-1-phosphate + starch ⇌ inorganic phospha

Hanes showed that the ' starch ' (85 per cent. of which separated from the digest in granular form) had the properties of an amylose rather than those of whole starch and the view that potato phosphorylase, when isolated from the tuber, effected the synthesis of only the unbranched component of natural starch was confirmed by the submission of a sample of Hanes's granular synthetic starch to end-group assay.[18] The average chain-length calculated from this assay (80-90 glucose members) is not so great as that found later for amylose separated from potato starch by the thymol method[19] and it is very probable that the synthetic starch contained a small proportion of amylopectin. The presence of a proportion of the branched chain component in the synthetic product would account for the observation that the latter gives an X-ray pattern very similar to that of whole potato starch (see Fig. 4:1.) rather than the V-pattern given by precipitated amylose.[20] The fact remains that ' synthetic starch ' is predominantly an amylose and the simultaneous synthesis of a small proportion of amylopectin is to be ascribed to the probable presence of the branching enzyme (Q-enzyme) in the phosphorylase preparation.[13]

There is thus a striking difference in the modes of action of phosphorylase when it is functioning in the living plant and when it is separated from organised cellular tissue. The starch synthesised in the plant contains 80 per cent. amylopectin whereas that synthesised *in vitro* by purified phosphorylase consist mainly of amylose.

The difference is even more impressive in the case of the animal phosphorylase. The phosphorylase system in intact muscle effects the synthesis of red-staining, highly-branched glycogen. On the other hand, crystalline phosphorylase extracted from muscle, acting upon glucose-1-phosphate (the Cori ester), does not bring about the synthesis of glycogen. Instead, a blue-staining unbranched amylose having an average chain-length of 200 glucose members is formed.[21]

The conclusion is inevitable that, in both cases, phosphorylase is only a part of the enzyme system concerned with the *in vivo* synthesis of polysaccharide from Cori ester. In living tissue the synthetic action of phosphorylase must be supplemented by some other enzyme which is capable of introducing cross linkages between the unbranched chains of glucose residues.

Such a factor was shown by Cori and Cori[22] to be present in liver and heart. When an extract of one of these tissues, which in itself no action on Cori ester, was added to a crystalline phosphorylase digest with the ester, the product was not

an amylose but instead there was obtained a polyglucose having the properties of glycogen.

Four-fifths of potato starch consists of amylopectin in which about 5 per cent. of the glucosidic linkages are branch linkages, i.e. are other than 1 : 4-glucosidic linkages. There is evidence that these branch links are probably 1 : 6-glucosidic (see Chapter 3), and that they are incapable of being formed by phosphorylase alone. The isolation of the branching enzyme of the potato was reported in 1944,[23] when it was shown that the action of a mixture of phosphorylase and the newly isolated factor (referred to as Q-enzyme) on glucose-1-phosphate resulted in the synthesis of a typical amylopectin apparently unaccompanied by amylose.[13] The synthesising function of Q-enzyme appears to be confined to the establishment of polymeric bonds (i.e. 1 : 6-glucosidic links) between pre-existing chains of 1 : 4-linked glucose members. Unless it is acting in conjunction with phosphorylase, Q-enzyme cannot utilise the Cori ester as substrate and indeed it is doubtful if Q-enzyme participates at all in the phosphate-interconversion system. Certainly it is not a phosphorylytic enzyme. For Q-enzyme plays a dual role in starch metabolism, it both synthesises (as described above) and degrades. The degradation, however, partakes more of the nature of amylolysis than phosphorolysis since the reaction proceeds in the absence of inorganic phosphate.[14] Nevertheless Q-enzyme is not an amylase of either of the known types. It is readily distinguished from these by a comparative study of the hydrolysis of amylose by the three enzymes and by its different stability towards heat and alcohol treatment.

Potato juice contains true amylases (mainly α-amylase) and much of the dubiety which still exists with regard to the hydrolytic function of Q-enzyme is due to the experimental difficulties involved in the complete elimination of amylase from Q-preparations. It is regarded as significant, however, that the purest specimens of Q-enzyme were able to convert amylose, which stains blue with iodine, into a permanent red-staining product without the concomitant liberation of either glucose or maltose. The red-staining product appears to be resistant to further action by Q-enzyme and it is this fact which distinguishes the mode of action of Q-enzyme most clearly from the initial stages of the action of α-amylase on amylose. α-Amylolysis very rapidly results in a complete loss of the power to stain with iodine.

What is the red-staining product of the action of Q-enzyme on amylose? An answer to this question would go far towards the elucidation of the complex problem of the interconnection which exists between the amylase and phosphorylase systems.

found, for example, that for the initial rates of synthesis to be the same, the amount of amylose needed as starter was 20 times as great as the amount of glycogen required. Now, assay shows that the proportion of end-groups in amylose is about one-twentieth of that in glycogen. A very clear indication is thus afforded that the dominating factor in the functioning of a polysaccharide starter is the number of non-reducing end groups it presents to the synthesising enzyme.

Accepting this hypothesis, it is obvious that the submission of amylose (itself an inefficient activator) to any hydrolytic process which will increase its end-group concentration per unit weight will also increase its efficiency as an activator. This is found to be the case when amylose is hydrolysed with acid.[30, 25] Its ability to activate potato phosphorylase increases as hydrolysis proceeds until the achromic point is reached. Thereafter, its activating power diminishes and disappears when complete hydrolysis to glucose is achieved. Cori *et al.*[28] state that activation reaches a peak when 30 per cent. of the amylose has been hydrolysed and that at this point the average chain-length of the dextrins produced is about 5 glucose units.

The effect of enzymic hydrolysis of amylose or amylopectin on its activating power will depend on the type of amylase used. Thus, β-amylolysis of either of the components of starch does not increase the number of chain-ends and would therefore not be expected to cause any increase in activating power. On the other hand, α-amylase brings about a fragmentation of the chains by scission of 1 : 4 glycosidic links and as hydrolysis proceeds the number of activating chain-ends increases. The effect of α-amylolysis and of acid hydrolysis on activating power would therefore be of similar character.

End-group concentration is not the only factor to be considered in respect of the activating power of a polyglucose in synthesis. Another factor, which becomes increasingly important as the molecular size of the starter is diminished, is the actual number of glucose members in the activating chain. Maltose does not function as an activator but, according to the observations of Weibull and Tiselius,[31] maltotriose (a chain of 3 glucose members linked by α-1 : 4 links) has some activating power. Branched [illegible] sucrose and its [illegible] which the effective chain length is two, are would seem tha[illegible] in it but sucrose, the fr[illegible] detected field agree that the optimum length fructosan in th[illegible] ich acts as a starter in synthesis by deposited as sta[illegible] ascination t six glucose units. This is shown by Rivets wheat d[illegible] d the pa[illegible] on the acid hydrolysis products of support to this [illegible] in the leaf befo[illegible]

amylose. The cyclic Schardinger dextrins (6-7 glucose members) do not function as activators until the ring is opened by mild acid hydrolysis, from which observation Proehl and Day[32] concluded that activators of potato phosphorylase need not contain more than 6-7 glucose units in an unbranched chain.

It is immaterial whether the polysaccharide activator is of the branched or the unbranched type provided that it offers unbranched chains, each of five or more glucose members, terminating in non-reducing units. In dextrins derived from amylose the length of the activating chain is simply the number of glucose units between the reducing and non-reducing ends. In the amylopectin structural type, however, the activating chains constitute only part of the molecule. They are the 'outer' chains with free ends and the length of the chain concerned in activation is the number of units between the non-reducing end and the nearest branch point.

The Inter-relationship of Starch and Sucrose in the Plant.—It has already been stated that sucrose may perform two functions. In many plants, particularly those which are not starch-forming, sucrose constitutes the chief carbohydrate reserve material. Together with its hydrolytic products, sucrose is also the form in which carbohydrate is translocated in most, and possibly in all, plants. The constituent common to starch and sucrose is glucose and the conversion of starch into sucrose, such as occurs for instance in the ripening of apples, must be linked with the enzymic processes of starch degradation which we have discussed above. The fundamental part played by glucose-1-phosphate in starch metabolism points to the conception of this hexose-phosphate as the intermediate in the interconversion of starch and sucrose. If this is true, an enzyme system must exist which is capable of using glucose-1-phosphate as a substrate for the synthesis of sucrose. Such an enzyme has in fact been isolated and by its action on a mixture of glucose-1-phosphate and fructose, the synthesis of sucrose *in vitro* has been achieved for the first time (Doudoroff, Hassid and co-workers,[33, 34]). This new phosphorylase is prepared from a bacterial source and Doudoroff reports that a search for its presence in the higher plants has so far been without avail.[35] This does not necessarily mean that sucrose synthesis in plants takes place b[illegible] [illegible]hes the mode o[illegible] or that glucose-1-phosphate is not involved [illegible]ial stages [illegible]nt [illegible] until a sucrose synthesising enzyme has been [illegible]is very [illegible]these the hypothesis remains tentative.

Another question of equal importance and f[illegible] [illegible]ith iodin[illegible] the origin of the fructose moiety of sucrose a[illegible] [illegible]f Q-enz[illegible] S[illegible] [illegible]ar towa[illegible] [illegible]nection [illegible] [illegible] and phosp[illegible] [illegible]ase system[illegible]

in these metabolic changes. The bacterial phosphorylase mentioned above is highly specific with respect to the hexose phosphate but not to the ketose constituent of the substrate. If α-glucose-1-phosphate is replaced by other hexose phosphoric esters no synthesis occurs, but the role played by fructose can be equally well taken by other ketoses such as L-sorbose or D-ketoxylose.[36] With L-sorbose and glucose-1-phosphate an isomer of sucrose results and the ketose residue is in the furanose form.[37]

Fructose occurs in the free state (when it is always associated with glucose) and as a constituent of sucrose and of certain tri- and tetra-saccharides. But, in addition, polysaccharides constituted solely of fructose residues are found in many plants. These polyfructoses, or fructosans, appear to function in a manner analogous to starch as carbohydrate reserve materials. Inulin, for instance, occurs as a soluble reserve in many plant species and especially in members of the *Compositae* such as dahlia, artichoke, chicory or dandelion. In the storage organs of these plants, the fructosan occurs alone, but in some species, in the snowdrop bulb for instance, inulin and starch are laid down side by side.[38] In inulin, the fructofuranose units are joined by 1 : 2-linkages; in other fructosans, the so-called levans, the linkages are 2 : 6-glycosidic. Levans have been found in the higher plants but they are most frequently encountered as products of bacterial metabolism.

It is a fact of great significance that combined fructose is found invariably in the furanose form. The free fructopyranose associated with glucose and sucrose in tissue fluids might also be regarded as an accidental product of the hydrolysis of sucrose or fructosans, the active metabolic form possessing the five-membered furanose ring structure.

There is botanical evidence of the interdependence of starch, sucrose and fructosan in the plant. In a study of the genesis of carbohydrates in wheat, Colin and Belval[39] observed the development of a fructosan in the stem which was coincident with the appearance of the ear and the laying down of starch in the seed. The leaves at no time contained either starch or fructosan but only sucrose and its hydrolytic products, glucose and fructose. It would seem that carbohydrate is translocated from the leaf as sucrose, the fructofuranose part of which is converted into fructosan in the stem at the same time as the glucose part is deposited as starch in the grain. Barnell[40] made observations on Rivets wheat during its growth from March to July which lent support to this view. The maximum sucrose content was found in the leaf before the middle of March; in the leaf-sheath at the

beginning of June and in the stem and head in the first week of July. Fructosan content was especially high in the young head but fell rapidly as starch formation proceeded in the developing ear. It should be pointed out, however, that Archbold and Datta[41] maintain that the sugars in the stem (of the barley plant) are not the precursors of the starch in the ear. The conversion of fructosan into sucrose is observed in artichoke tubers during winter storage.[42] In the later stages of the growth of the apple there is an extensive conversion of ' glucose ' units into ' fructose ' units, which may be in effect the conversion of starch into sucrose.[43]

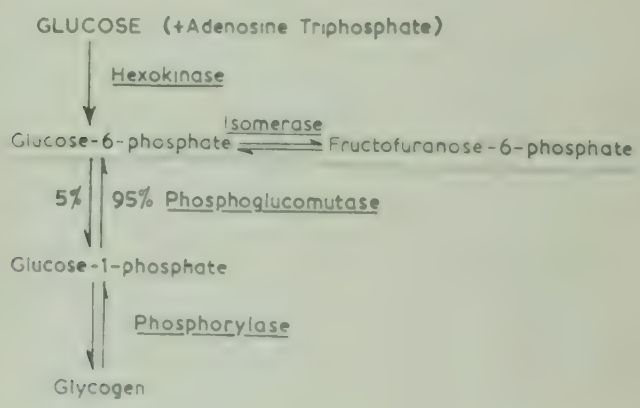

Fig. 2 : 2.

Evidence of this kind could be multiplied and it is very clear that glucopyranose and fructofuranose fulfil practically identical functions in plant economy. These interchangeable units are found combined together in sucrose.

How is the interconversion of glucopyranose and fructofuranose brought about in living tissue? At least one answer to this question is already known as a result of the great progress that has been made in the study of the mechanisms of phosphate-exchange, particularly in yeast fermentations and muscle metabolism. Lohmann[44] showed that yeast contained an enzyme, isomerase, which catalysed the reversible reaction :

glucopyranose-6-phosphate $\rightleftharpoons$ fructofuranose-6-phosphate.

The equilibrium mixture, of which the glucose phosphate constitutes two-thirds, is readily formed from either glucose or fructose by the agency of another enzyme present in yeast and

muscle and named hexokinase.[45] Furthermore, the connection between this enzymic equilibrium and the phosphogenesis of starch or glycogen became clear when an enzyme was isolated, from pea seeds[17] and from muscle,[46] which catalysed the interconversion of glucose-6-phosphate and glucose-1-phosphate. Hanes[17] describes this enzyme system as phosphoglucose-conversion enzymes while Colowick and Sutherland[46] use the term phosphoglucomutase. The conversion of free glucose into glycogen (or starch) is now a laboratory accomplishment and is represented by Colowick and Sutherland as in Fig. 2 : 2.

It is not to be argued from Fig. 2 : 2 that free glucose is an essential starting material in the synthesis of glycogen (or of starch). The glucose-6-phosphate may originate from other sources than glucose itself. It could arise, for instance, from phosphopyruvic acid by a reversal of the reactions of the Embden-Meyerhof conception of lactic acid production in muscle.

There would appear to be two mechanisms for the biological synthesis of the furanose form of fructose. The one is illustrated by the bacterial synthesis of sucrose from fructopyranose and glucose-1-phosphate ; the other by the isomerisation of glucose-6-phosphate to fructofuranose-6-phosphate.

The fructose phosphate (in which the sugar ring is necessarily five-membered) is biogenetically related, through glucose-6-phosphate, to glucose-1-phosphate. Inasmuch as the last-named ester provides the basis for the synthesis of both starch and sucrose, it might be expected that the fructose ester would constitute the connecting link between starch on the one hand and fructosan (and the ketose component of sucrose) on the other. The available evidence does not, however, support this idea. Indeed, Doudoroff[47] has shown that fructofuranose-6-phosphate cannot be used as a substitute for fructose in the bacterial synthesis of sucrose. Furthermore, in such syntheses of fructosan as have been described (and here again the enzymes concerned have been found only in bacteria), phosphorylation plays no part. In the synthesis of levan by *Aerobacter levanicum*, for instance, the fructose residues are already in the furanose form in the substrate, which may be either sucrose or raffinose. Phosphorylated fructose is not utilised.[48]

The alternative mechanism makes the synthesis of sucrose the stage at which the ring-change from pyranose to furanose is effected, although the reversible transformation of aldose into ketose may still be brought about by the isomerisation of the 6-phosphates. This being so, an additional step in the series of transformations will consist in the hydrolysis of fructofuranose-6-

phosphate and the liberation of fructopyranose. This hydrolysis might be a function of the widely distributed enzyme, phosphatase.

In conclusion, let it be admitted that many gaps exist in our knowledge of the transformations undergone by carbohydrates in the life cycle of a plant and that the biological picture of the functions of starch and its relationship to the other carbohydrate participants is still blurred and out of focus. Nevertheless the main outlines are becoming clearer. It is thus seen that the centre of the metabolic stage is occupied by the disaccharide, sucrose. When sucrose is produced in excess of immediate requirements, it is stored in appropriate organs either as such (e.g. in beet) or by being transformed into starch (in the potato, cereals, etc.) or into polyfructofuranoses (e.g. in dahlia). The starch and fructosans are immobilised in the storage cells by their high molecular weight but sucrose is free to diffuse through the cell walls and is thus the chief form in which carbohydrate is translocated. Both starch and fructosan, however, play more than the purely passive role of stored reserve materials. Many examples have already been cited which show that 'temporary' starch is available wherever active carbohydrate utilisation or assimilation is in progress, as in germinating seeds or during the operation of the photosynthetic process in leaves. An interesting observation relevant to this subject was made by Daoud and Tadros.[49] The reserve carbohydrate in the ungerminated seeds of fenugreek or of carob is neither starch nor a fructosan but is a mannogalactan. Mature ungerminated seeds are starch-free but germinated seeds contain starch (and some fructosan) and this increases with the progress of germination. Sucrose is also formed during germination but the appearance of starch precedes that of sugars by some 36 hours. When the radicles of ungerminated seeds are removed and the residual seeds are incubated at the same time as intact seeds, neither starch nor sucrose appear in the radicle-less seeds but considerable amounts appear in the intact seeds.

The investigation of metabolically 'active' starch, such as occurs in the leaf, has been neglected in the past, possibly because of the experimental difficulties involved, but one feels that such an investigation would contribute more to a solution of the problems still outstanding than would a continued concentration on the chemistry of 'depot' starch.

Supplement to Chapter 2. There have been extensive developments in the field covered by Chapter 2 in the five years which have passed since it was written. It seems desirable therefore that some of these advances in knowledge should be incorporated in this supplement.

Amylolysis.—Perhaps the most significant development in the period under review is the isolation of enzymes which are capable of hydrolysing the branch (i.e. α-1 : 6-glucosidic) links of amylopectin and of glycogen. The amylo-1 : 6-glucosidase from muscle (Cori and Larner[50]) performs this function with respect to glycogen while the R-enzyme, isolated by Hobson, Whelan and Peat[51] from potato and broad bean, hydrolyses the branch linkages of amylopectin which are not susceptible to the action of α- or β-amylase or of maltase. The *in vitro* conversion of both components of starch into glucose by enzymic hydrolysis is thus an accomplished fact.

The crystallisation of α-amylase from a number of sources (see Meyer and Gonon[52] for references) has accelerated the study of the mechanism of amylolysis as has the preparation of crystalline β-amylase from sweet potato[53] and from malt.[54] These more precise studies have confirmed the general picture given of the course of breakdown of the components of starch by the amylolytic enzymes. It has further become clear that maltotriose as well as maltose is formed in considerable amounts in the second stage of the action of α-amylase on amylose[55] and amylopectin.[56] Maltotriose is hydrolysed to maltose and glucose by β-amylase but not by salivary α-amylase.

Synthesis.—The investigation of amylosynthease has been taken up anew by Kobayashi and Maruo who have shown that it is not a synthesising enzyme but rather is a de-branching enzyme only. For this reason the authors propose to change its name to 'isoamylase'. The Japanese authors have agreed that isoamylase from yeast and R-enzyme from potato are very probably identical.[57] It is not therefore an enzyme of the Q-type.

Phosphorolysis and Phosphogenesis.—The branching enzyme of plants (Q-enzyme) has been the subject of close investigation over the past five years and the functions ascribed to it in the text have been fully confirmed (see Barker, Bourne, Wilkinson and Peat.)[58] The scheme of synthesis of amylopectin tentatively proposed in 1945, and portrayed in Fig. 2 : 1, has required some modification as the investigation of Q-enzyme proceeded. It is not necessary to postulate a duel function for Q-enzyme implying the intermediate formation of 'pseudo-amylose' from either amylose by Q-enzyme action of glucose-1-phosphate by the agency of phosphorylase. Indeed, a consideration of the energy changes involved is against such a supposition. Hobson, Whelan and Peat[59] produced evidence that Q-enzyme cannot link together linear chains as short as those of pseudo-amylose and that the *only* substrate on which it can act consist of linear chains approaching

in length those of natural amylose. On this view, the conversion of amylose into amylopectin by Q-enzyme consists in the *simultaneous* scission of 1 : 4-links (an amylose) and synthesis of 1 : 6-links to form amylopectin, i.e. Q-enzyme is a transglycosidase and the energy of synthesis of a branch-link is derived from the contemporaneous scission of a linear chain link. This action of Q-enzyme is, in a practical sense at least irreversible[58] and it follows that when a mixture of phosphorylase and Q-enzyme act on the Cori ester, they act independently according to the scheme :

Glucose-1-phosphate
⇅ Phosphorylase
Amylose (+ H_3PO_4)
| Q-enzyme
↓
Amylopectin

It should be emphasised that phosphate-transfer plays no part in the action of Q-enzyme.[60]

Polysaccharide Activators.—The Cori hypothesis[28] that polysaccharides act as primers in the direction of synthesis (by phosphorylase) because they enter stoichiometrically into the reaction has been substantiated by later work, mainly by the Cori school, on the muscle enzyme. A good account of the kinetics of the reaction is given by Hestrin.[61]

The minimum length of a priming chain is given in the text as being probably 5-6 glucose units although reference is made to the estimate of Weibull and Tiselius[31] of three glucose units. It has now been shown that the latter estimate correctly represents the minimum priming chain length. An absolute separation of a mixture of short-chain dextrins derived from amylose by acid hydrolysis has been achieved by partition on a charcoal column. In this way pure specimens of glucose, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose were obtained. The pure maltotriose (as well as the higher saccharides) possessed definite priming activity and could be used as the sole primer in the synthesis of amylose by phosphorylase.[62]

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Chapter 3

THE CHEMICAL EVIDENCE FOR THE STRUCTURE OF STARCH

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Within recent years, considerable progress has been achieved in the intrinsic chemical and biochemical problems which are associated with the molecular constitution of starch. Perhaps the most significant advance has been the separation and conclusive identification of two chemically distinct components, namely, amylose and amylopectin from whole starch, although it has not yet been conclusively ascertained whether they exist in the starch granule as two different chemical entities or combined one with another. As will be seen later, highly selective methods have been developed for the isolation of amylose and amylopectin, and many of the constitutional studies previously carried out on whole starch have been repeated on each of these fractions. An extensive study was clearly necessary of the proportions of amylose and amylopectin present in various starches and of the type of structure present in each. This has been facilitated by recently developed methods of analysis; for example, the blue value method of Hassid and McCready and the potentiometric iodine titration method of Bates, Rundle and French for the estimation of amylose, and by the new methods of 'end-group assay', including the quantitative periodate oxidation method developed by Halsall, Hirst and Jones and quantitative analysis of sugars and their methylated derivatives by use of paper partition chromatography.

It is clear already that different starches contain very different amounts of the two components and that the ease of separation varies from one starch to another. In this chapter only the chemical evidence for the structure of starch will be considered, and except in so far as structural problems are involved, the chemistry of the derivatives of starch will be omitted, despite their great chemical importance in commerce. The structural implications derived from investigations of the degradation of starch by enzymic action are given elsewhere in this volume (see

Chaps. 18, 20–23). Initially, the authors propose to describe the chemical evidence for the structure of starch prior to 1939, in particular, the invaluable contributions of Haworth and Hirst, and then to present the evidence upon which modern views concerning the structure of starch and its components, amylose and amylopectin, are based.

Starch occurs in plants in the form of granules, the size, shape and striations of which are characteristic for the variety of starch, thus providing a microscopical method of identification of the biological origin of the starch (see Table I). The outer membrane of the starch granule, which is usually perforated,[1] is much more resistant to enzyme action than the starch enclosed within. Rupture of this membrane by grinding, heating, etc., facilitates dispersion of the starch.[2] Wood starch (from oak heartwood) is also granular, but in this case no resistant outer membrane has been encountered.

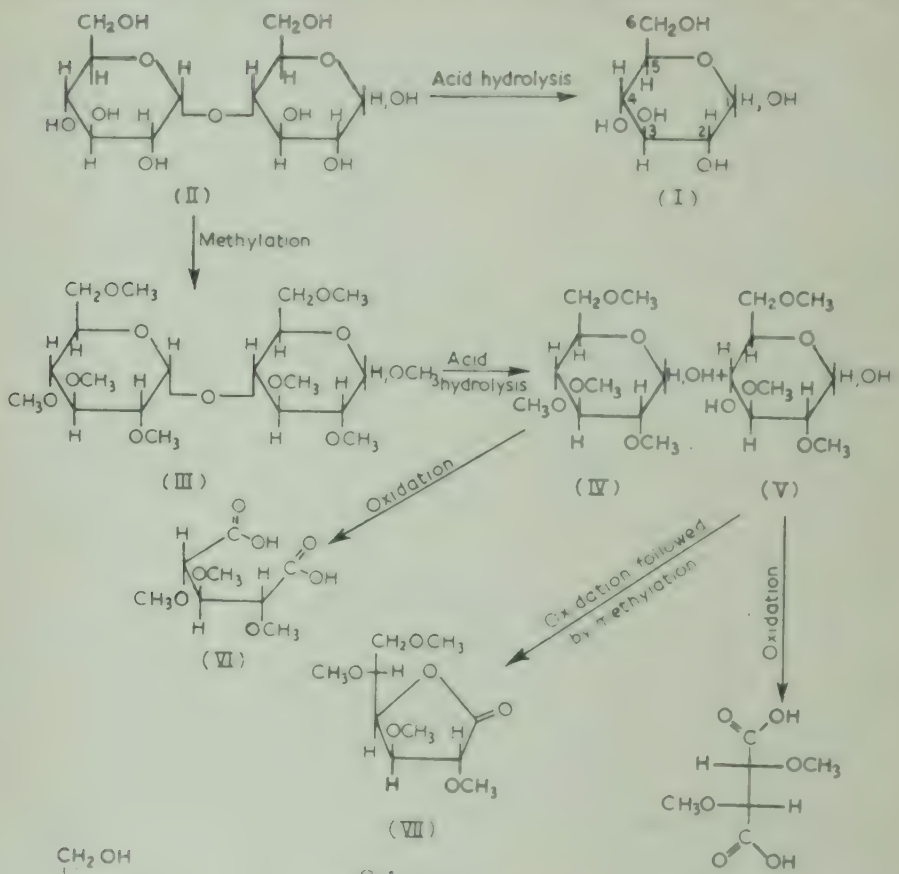
TABLE I

Starch	*Diameter (mm.)*	*Volume (mm.^{3})*
Potato . . .	$0{\cdot}04 \times 10^{-6}$	17×10^{-6}
Rice . . .	$0{\cdot}004 \times 10^{-6}$	$0{\cdot}034 \times 10^{-6}$
Maize . . .	$0{\cdot}014 \times 10^{-6}$	$1{\cdot}5 \times 10^{-6}$

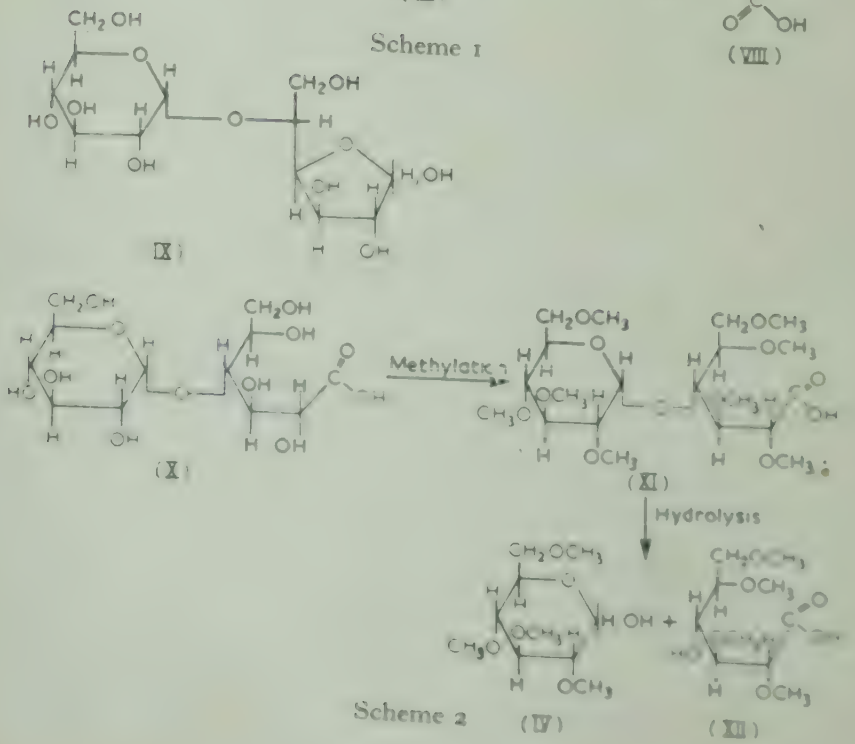
Starch is often associated with small quantities of non-carbohydrate materials such as fatty acids,[3] silicic acid,[4] phosphates[5] and proteins.[6] At one time it was considered that these materials existed in organic combination with starch, thereby constituting a part of the molecular structure. It has been shown recently, however, that these materials, with the exception of phosphate ester groups[5] and probably protein, exist in starch as extraneous impurities which can be eliminated by careful purification. For example, Schoch[7] has demonstrated that the fatty acid material present in cereal starches can be removed without hydrolytic degradation of the starch by extraction with a suitable water-miscible fat solvent such as methyl alcohol or dioxan. He also showed that this procedure removed the greater proportion of the phosphoric acid derivatives from the cereal starches but only a small proportion from potato starch. This result is in agreement with the experimental findings of Posternak[5] who found that starches stored in rhizomes and tubers contained phosphates combined in ester form with the hydroxyl groups on C_6 of glucose residues in the starch. Earlier claims by Ling and Nanji that

starch contains combined silicic acid have not been substantiated by more recent work. The function of the small amount of protein in starch is uncertain but the suggestion[6] has been put forward that it represents some residue derived from the enzyme originally responsible for the synthesis of the polysaccharide to which it still remains in attachment. It is clear, therefore, that these minor constituents have no significance in connection with the general chemical structure of starch (see Chapter 6).

It has long been known that the main product resulting from the hydrolysis of whole starch by acid is *D*-glucose (I), which may be recovered practically quantitatively. The mode of linkage of the glucose residues present in starch was suggested by early experiments[8] in which maltose was isolated from the degradation products of starch by the action of acid or by treatment with an enzyme present in ungerminated cereals, namely, β-amylase. Maltose was shown by Irvine and Black and by Cooper, Haworth and Peat[9] to have one of two formulae (II or IX), in the following manner. On methylation, maltose yields heptamethyl methyl maltoside (III), a syrup which may be purified by distillation in a high vacuum. This product, on hydrolysis in acid solution, affords equimolecular proportions of 2 : 3 : 4 : 6-tetramethyl *D*-glucose (IV) and 2 : 3 : 6-trimethyl *D*-glucose (V) (both of known constitution). The structure of the former sugar was proved by Hirst[10] by oxidation with nitric acid, and the isolation of *inactive xylo*-trimethoxyglutaric acid (VI), thus demonstrating the presence of a pyranose ring in the original sugar. Haworth, Learner and Long[11] found that the trimethyl *D*-glucose (V) on methylation gives 2 : 3 : 4 : 6-tetramethyl methyl *D*-glucoside which contains the pyranose ring structure, and therefore the original trimethyl glucose possesses a hydroxyl group on C_5. The trimethyl sugar on oxidation yielded a furanolactone which, on methylation with silver oxide and methyl iodide, yielded 2 : 3 : 5 : 6-tetramethyl *D*-gluconolactone (VII), possessing a hydroxyl group on C_4. The trimethyl sugar is therefore 2 : 3 : 6-trimethyl *D*-glucose (V), possessing hydroxyl groups on C_4 and C_5; further evidence for the structure of this sugar was afforded by oxidation with nitric acid to give *L*-dimethoxysuccinic acid (VIII). Maltose therefore consists of two *D*-glucopyranose units linked one to another in such a manner that the reducing group of one such unit is joined to *either* C_4 *or* C_5 of the other molecule (II; IX). In (II), two glucopyranose units are joined by a 1 : 4 glycosidic linkage, C_5 being engaged in pyranose ring formation in each unit. In the second formula (IX), however, the units are joined by a 1 : 5 glycosidic linkage, the units possessing pyranose and furanose



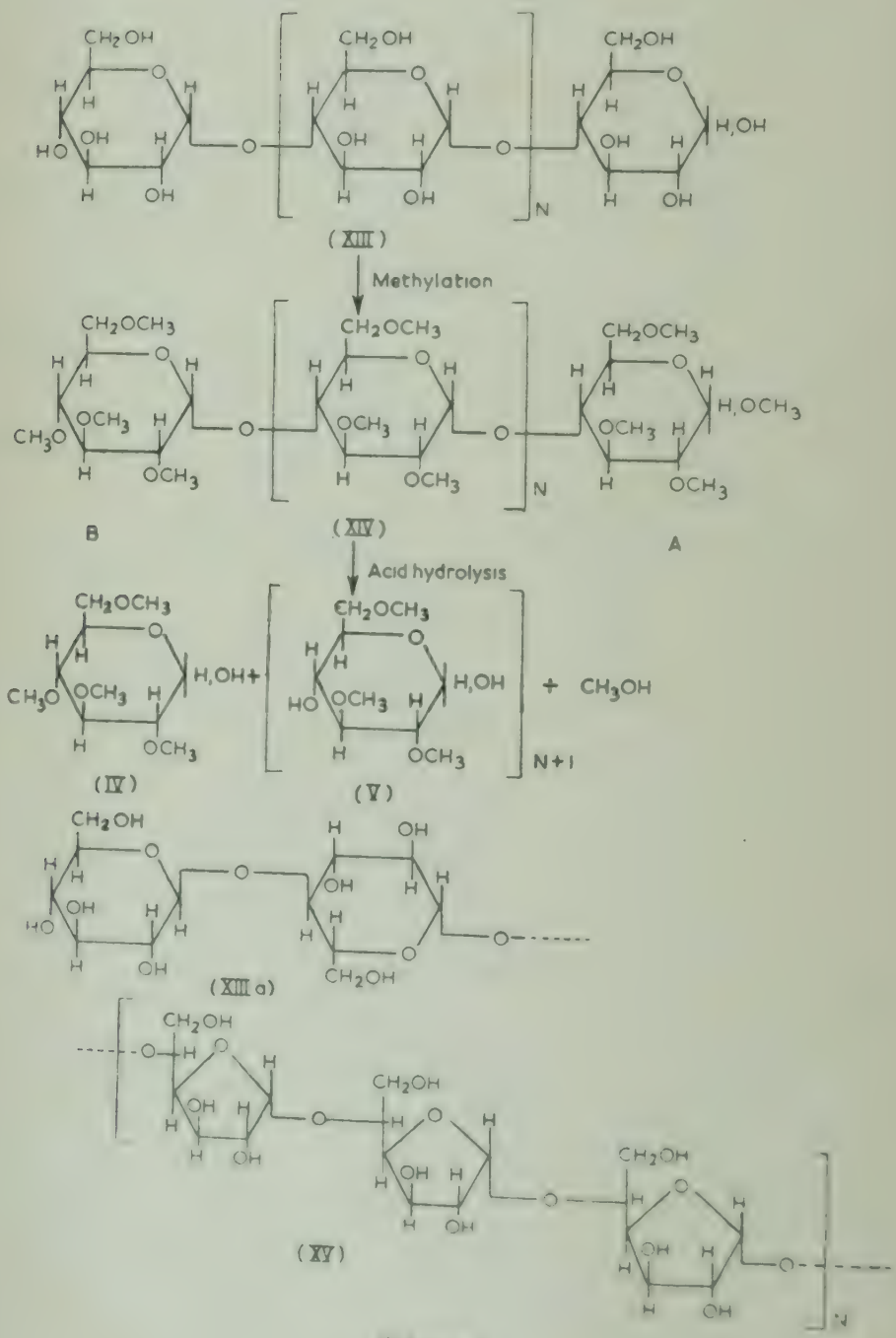
Scheme 1



Scheme 2

ring structures respectively. (II) was proved[12] to be the correct structure for maltose from a study of the reactions of maltobionic acid (X) obtained by oxidation of maltose with bromine water. Maltobionic acid on methylation with methyl sulphate and caustic soda yields an octamethyl derivative (XI), which on hydrolysis affords an equimolecular mixture of 2 : 3 : 4 : 6-tetramethyl *D*-glucose (IV) and tetramethyl *D*-gluconic acid (XII). The latter is readily converted into the known 2 : 3 : 5 : 6-tetramethyl *D*-glucofuranolactone (VII). Since this product possesses only one hydroxyl group, at C_4, it follows that the glucosidic linkage between the glucose and gluconic acid derivatives must be between C_1 and C_4 respectively. A consideration of the optical rotation of maltose indicates that the glucopyranose units are linked by an α-bond from the glucosidic (reducing) hydroxyl on C_1 of one glucose residue to the hydroxyl group on C_4 of the second.

Further structural evidence was obtained by Irvine and Macdonald[13] and by Haworth, Hirst and Webb[14] from a study of the products obtained by methylation of potato starch. By repeated treatments with methyl sulphate and alkali, the hydroxyl groups are replaced by methoxyl, giving a methyl ether derivative which is characterised by optical rotation and viscosity measurements. Hydrolysis of the fully methylated potato starch (XIV) gives 2 : 3 : 6-trimethyl *D*-glucose (V) in high yield, together with a small proportion of 2 : 3 : 4 : 6-tetramethyl *D*-glucose (IV). These products may be separated by fractional distillation under reduced pressure. The identification of the former product shows that the hydroxyl groups on C_4 and C_5 in the glucose molecule are protected in some manner[15] in starch and indicated that starch consists, in the main, of *D*-glucopyranose residues joined by 1 : 4-linkages (XIII)[16]. It is possible, however, that the glucose residues are present in the furanose form, which in this case would be joined by 1 : 5-linkages (XV). However, the isolation of 2 : 3 : 5 : 6-tetramethyl *D*-gluconolactone (VII) after oxidation and methylation of the products of acetolysis of methylated starch provided the final proof that successive units are joined mainly by 1 : 4-α linkages (XIII)—a fact that does not necessarily follow from the isolation of maltose from starch by the action of β-amylase, since enzymes are capable of both synthesis and degradation. Since positions 1 and 4 are concerned in the junction of successive glucose residues, the free hydroxyl group on C_5 must therefore have been involved in ring formation of the glucose molecule, indicating that the glucose is present in starch in the form of the relatively stable 6-membered pyranose ring.


(XIII)
Methylation
B
(XIV)
A
Acid hydrolysis
(IV)
(V)
+ CH3OH
N+1
(XIII a)
(XV)

Scheme 3

The presence of α-linkages was established by Freudenberg from a study of the optical rotatory power of starch.

These and other facts indicate that whole starch contains chains of glucopyranose residues, united mainly by 1 : 4-α-linkages in contra-distinction from cellulose, where the junctions are of the 1 : 4-β-type (XIIIa). The isolation of a small quantity of 2 : 3 : 4 : 6-tetramethyl *D*-glucopyranoside[17] from the products of methanolysis of methylated starch indicates, however, that these chains are of a finite length. If the glucose residues present in starch are united in such a manner, the terminal glucose units of the chain may be distinguished one from another as their methyl ether derivatives. An inspection of (XIV) shows that ' reducing ' end group (A) yields on acid hydrolysis 2 : 3 : 6-trimethyl *D*-glucose (V), whereas the non-reducing end group (B) affords 2 : 3 : 4 : 6-tetramethyl *D*-glucose (IV), which may be determined by fractional distillation of the scission products of methylated starch and an examination of the physical properties of the resultant fractions. It is clear that the non-reducing end group gives a measure of the average chain length of starch; this method is referred to as ' end group assay '. The method suffers from a number of disadvantages in that it involves the use of relatively large quantities of material, that the distillation procedure requires relatively high temperatures which invariably cause some decomposition of the methyl glycosides and that it is extremely difficult to effect a complete separation of the methyl glycosides. In order to overcome the complications encountered in the course of fractional distillation, special methods had to be devised to obtain accurate results.[32, 107] Other procedures have also been elaborated. For example, the methanolysis products of methylated starch may be separated by chromatography on a column of alumina, using ether as the eluting agent.[19] A similar method has been described by Norberg, Auerbach and Hixon,[20] who used benzene to elute the methylated sugars and followed the separation by use of ultra-violet light; an almost quantitative recovery of the methylated sugars was obtained. Considerable success has been obtained by the application of partition methods to the separation of these closely related methylated sugar derivatives. Macdonald[21] has calculated the partition coefficients of 2 : 3 : 4 : 6-tetramethyl glucose and 2 : 3 : 6-trimethyl glucose between chloroform and water, and has shown that they differ by a factor of 100. Acting on this suggestion, Bell[22] has developed a simple partition procedure for the assay of 2 : 3 : 4 : 6-tetramethyl glucose. Brown and Jones[19] have described a similar partition procedure for the separation of the methyl glycosides of

'tetra'- and 'tri'-methyl glucoses; the method is dependent upon the fact that the fully methylated sugar glycosides possess a higher partition coefficient between water and petroleum ether than the partially methylated sugar glycosides. Later, Bell applied the method of partition chromatography to the separation of the hydrolysis products of methylated starches and thereby developed an elegant procedure for the assay of tetramethyl glucose.[64] The separation is achieved by counter-current partition between first chloroform and then chloroform-*n*-butanol (the mobile phases) and a column of silica gel-water (the stationary phase). Using this technique, Bell has obtained an absolute separation of the tetra-, tri-, and di-methyl glucoses obtained on hydrolysis of as little as 1 gm. of methylated starch.[64] The methylated sugars may also be separated on the micro-scale by partition chromatography on sheets of filter paper, the reducing sugars being detected by such spray reagents as ammoniacal silver nitrate[108] or *p*-anisidine hydrochloride.[109] After separation, the methylated glucose derivatives may be extracted from the paper chromatogram and estimated quantitatively by oxidation with alkaline hypoiodite, or colorimetrically,[111] thus affording a method for the quantitative micro-estimation of the mixture.[108] The methylated sugars may also be separated by partition chromatography on columns of powdered cellulose.[109]

Concurrent with these advances in the methods of end-group assay, improvements in the methylation procedure have been devised. The use of liquid ammonia with sodium and methyl iodide has been described for the methylation of starch.[23] Methylation by this method has been criticized by Freudenberg and Boppel[24] and by Schorigina.[25] Bourne, Fantes and Peat,[26] however, find that whilst the methylation is accompanied by some degradation, the end-group determination is unaffected. Methylated starches obtained by exhaustive methylation with methyl sulphate and caustic soda are usually slightly under-methylated, but further treatment with sodium in liquid ammonia, ether or toluene and methyl iodide, gives the fully methylated product.[27]

From an examination of a large number of whole starches, the interesting fact emerges that starches from potato, maize, canna, rice, wheat, chestnut, banana and other sources, all yield approximately the same percentage of tetramethyl methyl *D*-glucopyranoside (4-5 per cent.) on methanolysis of the corresponding methyl ether derivatives (see Table II).

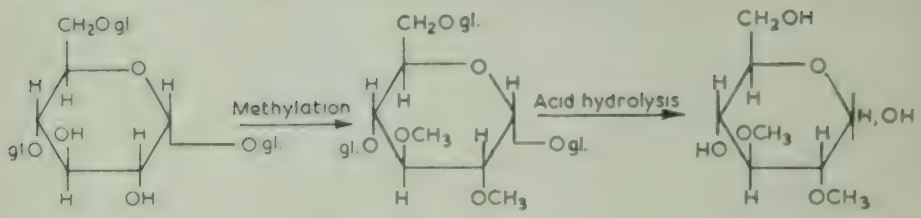
A similar proportion of 2 : 3-dimethyl *D*-glucose (XVII)[36, 37] was also isolated in a yield of about 5 per cent. from the hydrolysis products of methylated starches. The significance of this di-

TABLE II

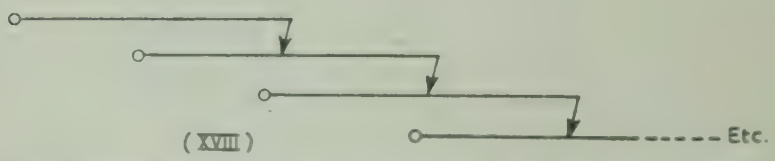
Source of Methylated Starch	*Average Number of Glucose Residues per Non-reducing End Groups*	*Reference*
Potato	25	(28)
Waxy Maize	20 ; 26	(30) (29)
Canna	27	(31)
Wheat	24	(32)
Horse-chestnut	28	(32)
Rice	26–30	(33)
Banana	24	(34)
Maize (Golden Bantam)	25	(35)
Sweet Potato	29-31	(30)

methyl glucose was doubtful, however. It could have arisen from a glucose residue, to which three other glucose residues were attached (XVI) in the original starch (which would indicate a branched chain structure) or alternatively, it could be an artefact, resulting from incomplete methylation of the starch or from the 2 : 3 : 6-trimethyl glucose by partial demethylation by the hydrochloric acid used for the scission of the methylated starch. The proportion of 4-5 per cent. tetramethyl glucose would indicate a terminated straight chain of 24 to 30 glucose units, having a molecular weight of about 5,000. It was soon recognised, however, that this view required modification since, an investigation of the molecular weights of various natural starches and their acetyl and methyl derivatives by osmotic pressure, ultracentrifugal and viscosity measurements gave a series of values ranging between 20,000 and 700,000, depending on the method of preparation of the acetyl and methyl derivatives.[33, 38]

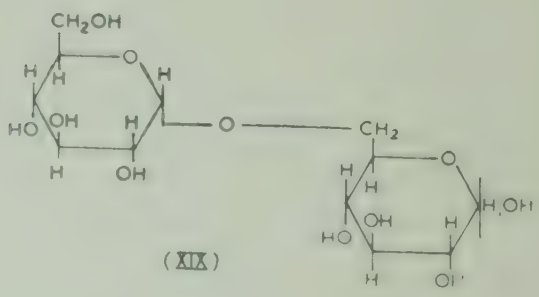
In the absence of oxygen and at low temperatures (20-30°), starch may be converted into a methyl ether derivative of high molecular weight. It had been noted by Haworth and his co-workers,[39] however, that repeated methylation under more vigorous conditions (in air) gradually reduces the molecular weight. Nevertheless, the apparent proportion of ' end group ' remains unchanged. Thus, in the course of seventeen methylations, the molecular weight of methylated maize starch was reduced from 700,000 to 38,000 (*ca.* 230 glucose residues), the percentage of ' end group ' being apparently the same before and after the treatment. It is clear that this and other observations of a similar nature are difficult to account for on any hypothesis of random hydrolysis of long *unbranched* chains of glucose residues such as were considered by Richardson for the structure of starch. Nevertheless, molecular weight determinations show that some

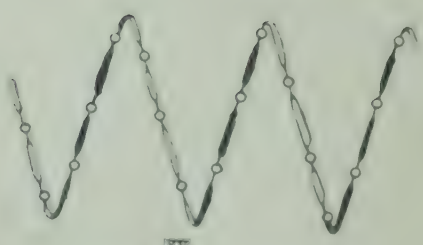


Scheme 4



Haworth and Hirst's laminated formulation for starch. Each horizontal line represents a chain of 24-30 glucose residues. The arrows represent junctions from the reducing end-group of each chain to a glucose unit in an adjacent chain. The circles represent the non-reducing end-groups.





(XX)

The Helical Amylose Structure.

degradation of the starch molecule must have occurred and a calculation shows that the increase in the percentage of 'end group' (from *ca.* 4 per cent. to 4·4 per cent.), which would result in random degradation of a molecule of molecular weight of 700,000 to one of molecular weight of 38,000 would be so small as to be within the limits of experimental error of the method of assay. In 1937, Haworth and Hirst suggested that starch had a laminated structure (XVIII), consisting of a number of 'repeating units', each unit consisting of a straight chain of 24-30 glucose residues joined by 1 : 4-α-linkages, and the repeating units being joined in such a manner that the reducing group of a glucose unit terminating one chain is linked glucosidically with a glucose unit in an adjacent chain.

Evidence concerning the presence of repeating units in the starch molecule has been obtained by Hirst and Young[33] in which a sample of methylated rice starch of molecular weight 5×10^5 was reduced to $2 \cdot 3 \times 10^4$ by hydrolysis with oxalic acid in aqueous methyl alcohol. After methylation of the degraded product to protect any hydroxyl groups exposed during the 'disaggregation', the percentage of end group was found to be identical with that found in the original specimen. The contrast between this 'disaggregation' reaction which produces no short chain dextrins and the normal random hydrolysis by acid is apparent. During 'disaggregation' the optical rotation of the methylated starch remains constant and on subsequent methylation it rises slightly; during acid hydrolysis, however, the optical rotation falls rapidly. The properties of a sample of 'disaggregated' methylated starch are compared with those of a normal sample of methylated starch and of methylated dextrins in Table III.

It is highly significant that the proportions of end group (one terminal residue for 30 glucose residues approximately) remain unchanged during the disaggregation, thus indicating that little, if any, degradation (splitting of 1 : 4-linkages) occurs during this operation.

There is evidence that the type of linkage which is split in the disaggregation of methylated starch is of the normal glucosidic type and is not a hydrogen bond, since the activation energy, calculated from the velocity of disaggregation at two different temperatures, is found to be 21,000 cals., while the activation energy required for the dissociation of polymers held together by hydrogen bonding is approximately 5,000 to 8,000 cals.[41] The disaggregation appears to be due to the hydrolysis of a glucopyranosidic linkage, since it proceeds under standard conditions

TABLE III

The Properties of Disaggregated Methyl Rice Starch

	Typical Normal Methyl Derivative	*Disaggregated Derivative*	*Typical Methylated Dextrin*
Molecular weight	200,000 (1,000 glucose units)	20,000 (100 glucose units)	1,000-4,000 (10-20 glucose units)
Average number glucose residues per non-reducing end group.	29-32 glucose units	31 glucose units	6-12 glucose units
$[\alpha]_D^{20°}$ in $CHCl_3$	+ 210°	+ 224°	+ 180-200°
Solubility in chloroform, acetone, etc.	Difficultly soluble	Very soluble	Very soluble
Melting point	*ca.* 200° decomp.	130° decomp.	

at about one-sixth of the rate of hydrolysis of methylated inulin, which contains chains of furanose sugar units. The above results provide further evidence for the laminated formulation (XVIII) of Haworth and Hirst, the unit chains being joined by linkages other than the 1 : 4-type. If the linkage is of the 1 : 6-type, then only the hydroxyls situated on C_2 and C_3 will be free, and consequently methylation followed by hydrolysis will result in the formation of a glucose derivative with methoxyl groups on C_2 and C_3. This would explain the presence of 2 : 3-dimethyl *D*-glucose (XVII) in the hydrolysis products of methylated starch[36, 37] and the isolation of a 1 : 6-linked disaccharide (*iso*-maltose) (XIX) from both the acidic and enzymic hydrolysis products of starch.[40] Myrbäck and Ahlborg[40] also isolated from the enzymic degradation of starch, a trisaccharide which on methylation and subsequent hydrolysis yielded a mixture of 2 : 3 : 4 : 6-tetramethyl *D*-glucose, 2 : 3 : 6-trimethyl and 2 : 3 : 4-trimethyl *D*-glucose, thus providing further evidence for the presence of 1 : 6-linkages in the starch molecule. At this stage, it was concluded, therefore, that some, at least, of the inter-unit bonds were of the 1 : 6-type.

In 1939, the problems associated with the structure of starch were complicated by the conclusive identification of two major components in the starch granule—amylopectin and amylose—both polymers of glucose, but displaying markedly different properties. Since the investigations described above apply to

whole starch, much of the work has been repeated using pure amylose and pure amylopectin. The amylopectin component has a high average molecular weight of the order of 10^6 and possesses a highly ramified structure, whilst the amylose component, which is usually present to a smaller extent, is of lower average molecular weight and consists of unbranched or slightly branched chains of glucose units. The first suggestion that starch was a mixture was made over 100 years ago,[42] but it was only with the introduction of novel methods of investigation that the final proof was obtained.[43] Prior to 1939, great difficulty was experienced in fractionating starch, owing to the practical problems involved in the preparation of completely dispersed starch solutions, and to the tendency of the colloidal particles to absorb impurities. The issue was further complicated by the fact that the viscosity relationships of starch solutions do not run simply parallel with the degree of separation achieved. It is now well known that within any group of defined polymeric structure, viscosity will depend on the degree of polymerisation. The viscosities alone, therefore, give no guide as to separation into structurally different components. The various modes of fractionation employed in the past have, in some cases, accomplished a separation or partial separation of the components of starch, although usually with attendant physical or chemical degradation. As a result, the whole subject, including nomenclature, became hopelessly confused until recent years when the position was clarified. The fractions of starch are characterised by their reaction with iodine solution and by the yields of maltose obtained on enzymic degradation by β-amylase. One portion of starch—amylose—was found to give an intense blue colour with iodine and to undergo practically complete degradation to maltose with β-amylase, whilst the other component—amylopectin—gave a purple colour with iodine and, with β-amylase, incomplete conversion to maltose was observed.

Samec[44] appears to have been the first worker to achieve a separation of amylose and amylopectin. He used an electrophoretic method to separate the components of potato starch; the amylopectin portion, which is one of the few containing organically esterified phosphoric acid, migrates and precipitates at the anode, whereas the amylose portion remains in solution. The method is not of general application, however, since the cereal starches, which do not contain organically bound phosphates and which are free from fatty acids, cannot be separated. The method also suffers from the disadvantage that retrogradation may occur and that local conditions of alkalinity or acidity

may be generated in the starch solutions by electrolysis with a consequent degradation of the starch.

A method which undoubtedly yielded fairly pure samples of amylose and amylopectin was first developed by Baldwin[45] and later used by Meyer.[46] In this method, the swollen but intact granules are leached with successive portions of warm water until no further soluble material is removed. The extract represents a crude amylose and the residue a crude amylopectin. This method is unsatisfactory, however, owing to the difficulty experienced in maintaining aseptic conditions and owing to the incomplete separation achieved. The preferential adsorption of amylose on cellulose (cotton) has also been employed for the separation of amylose and amylopectin.[47]

A more complete separation of whole starch into its components has been achieved by the use of novel methods developed initially by Schoch[48] in America and later by Peat[49, 50] and Higginbotham[51] in this country. In 1926, Alsberg observed that autoclaved starch solutions gave birefringent clusters with alcohol, and Schoch, in 1941, demonstrated that this crystalline precipitate was an amylose-alcohol complex. Schoch's original method for the separation of amylose from potato or corn starch consisted essentially of allowing the fat-free starch solution to cool very slowly in the presence of *n*-butyl alcohol or *n*-amyl alcohol, when the amylose separates as a crystalline complex which may be separated on the centrifuge. A later development of this method by Haworth and Peat[49] obviates the heat treatment by the use of thymol, which precipitates the amylose from cold solution. The amylose-alcohol complex may be 'recrystallised' by using the same procedure and the amylopectin is obtained as an amorphous mass by the addition of an excess of ethyl alcohol to the mother liquors. This method of separation depends upon the ability of the amylose component to react with hydrogen donors, such as, for example, cellulose,[47] butanol,[48] *n*-amyl alcohol,[43] fatty acids[56, 57] or thymol,[49] with the resultant formation of a crystalline complex. Higginbotham and Morrison[51] have shown that this separation, to be effective, must be carried out in very dilute solution. Other modifications of this method have been described by Whistler and Hilbert,[52] and Boise and Vallières.[54] A comprehensive survey of the fractionation of starch has been given by Schoch,[43] should further information be required (see also Chapter 6). An alternative method for the separation of amylose and amylopectin has been described recently by Bourne, Donnison, Peat and Whelan.[59] The method consists of absorbing potato starch on aluminium hydroxide and leaching the precipitate with boiling

water when the amylose passes into solution, little or none of the amylopectin dissolving. Using this technique, these workers have examined the amylose component of waxy maize starch, and they find that it resembles potato amylose and occurs to the extent of not more than 2 per cent. in the original starch[55] (see, however, Chapter 6).

An evaluation of the various methods of separation of amylose from amylopectin has been simplified by Bates, Rundle and French,[58] who observed that the blue colour given by starch solutions with iodine is due almost entirely to the formation of an iodine-amylose complex. They developed an elegant potentiometric method for the estimation of the iodine-binding capacity of starch, thus enabling the amylose content to be determined. Higginbotham and Morrison[51] have shown that, in a pure amylose, one glucose residue ($C_6H_{10}O_5$) will combine with 21·6 per cent. of its weight of iodine and that this figure is the same for amyloses isolated from several varieties of starch. Another method which has been widely used for the estimation of amylose and amylopectin has been described by Hassid and McCready[35] and is based on the difference of the absorption spectra of the iodine complexes. The amylose content of various starches determined by these methods were found to vary between 15-25 per cent. in the majority of cases (see Table VI)[30, 51, 58, 59, 60]. With few exceptions, these results were in good agreement with those obtained by the amylose-complex precipitation method. Certain 'waxy' starches such as waxy maize,[61] waxy rice and waxy sorghum, which were known to give a red colour with iodine, thus indicating the absence of amylose, were found to contain less than 1 per cent. amylose,[58] thus providing a useful source of amylopectin. On the other hand, the 'wrinkled' pea starches are rich in amylose;[60, 62] the 'Steadfast' variety is reported to contain 98 per cent. amylose[63] and other varieties are found to contain 50-60 per cent. amylose.[60] Improved methods for characterising the starch fractions in terms of intrinsic viscosity, iodine affinity, reducing power, and retrogradation of the amylose component have been described.[53]

Amylose.—Amylose is characterised by a deep blue coloration with iodine, by retrogradation from neutral aqueous solution and by the reaction with β-amylase preparations. In this reaction, providing that care is taken to ensure that the amylose does not retrograde from the reaction mixture, the enzyme converts amylose in almost quantitative yield into maltose.[40, 65]

In 1940, Hanes[67] observed that glucose-1-phosphate can give rise, under the influence of a potato phosphorylase, to a poly-

saccharide which separates in the form of granules, having physical properties similar to those of amylose. For example, the X-ray diffraction pattern of the synthetic substance, characteristic of crystalline material, was identical with that of amylose. Furthermore, the synthetic product gave a deep blue colour with iodine and was hydrolysed quantitatively to maltose by a β-amylase preparation. A chemical examination of synthetic amylose by Haworth, Heath and Peat[68] in England and by Hassid and McCready[69] in the U.S.A., shows that if potato phosphorylase was used as the enzyme, an unbranched polymer of 1 : 4-linked-*D*-glucopyranose residues with an average chain-length of the order of 100 glucose residues per non-reducing end-group was produced, but if synthesised by the agency of muscle phosphorylase it was of the order of 200 glucose residues.[70] The yield of tetramethyl methyl-*D*-glucopyranoside, resulting from the methanolysis of the methylated derivative of synthetic amylose, was approximately 0·2-0·5 per cent., a very much lower yield than that obtained from the methanolysis of methylated whole starch. Similarly, the methylated derivative of amylose, on methanolysis, yields very small quantities of tetramethyl methyl-*D*-glucopyranoside; Hess and Krajnc[71] obtained 0·4 per cent. yield from methylated potato amylose, Meyer and his collaborators[72] obtained 0·3 per cent. from methylated maize amylose and 0·4 per cent. from that of potato amylose, and Hassid and McCready[73] obtained 0·3 per cent. from methylated potato amylose. These figures provide mutual evidence for the presence of one non-reducing terminal group per 200-300 glucose residues in the amylose molecule. A sago amylose examined by Brown, Halsall, Hirst and Jones[30] was found to have an average chain-length of 250 glucose residues per non-reducing end-group.

The assumption is made that no degradation of the amylose occurs during methylation. The partition chromatography method described by Bell[64] for end-group assay of methylated starches is not applicable to the determination of the chain-length of amylose, since the end group, which is small, is accompanied by a syrupy impurity, which cannot be separated from it on a silica column.[26] Bourne, Fantes, and Peat[26] have modified the method for the end-group assay of methylated amylose and they have obtained values of 101, 191, and 187 for three different samples of amylose prepared from starch by thymol precipitation.

There is some dispute as to whether amylose is a branched chain polysaccharide or not—some workers consider that it is unbranched, while others are of the opinion that there are two non-reducing glucose end groups per amylose molecule since the

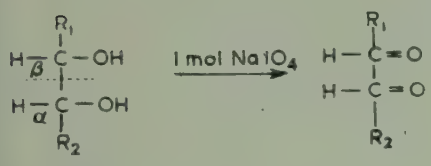
amount of end group isolated was twice that expected from the molecular weight of the molecule, as determined by osmotic pressure measurements.[65] Owing to the difficulty experienced in removing from the amylose fraction the last traces of amylopectin, which contains a relatively high percentage of end group (see later), considerable experimental difficulty may be encountered before this point is unequivocably decided, as appears, for example, from the work of Dombrow and Beckmann.[74] There seems to be little doubt, however, that amylose is a mixture of polymer homologues, consisting of chains with little or no branching with molecular sizes ranging from about 100 to 2,000 or more glucose residues.[75, 76] It is noteworthy, in this connection, that Meyer and his collaborators[77] have found that two fractions of maize amylose can be prepared by a combination of leaching and selective precipitation methods. A fraction of relatively low molecular weight (40,000), containing approximately 200 glucose residues, can be leached out and then a fraction of molecular weight 340,000, containing about 2,100 glucose residues can be obtained by subjecting the leached granules to the precipitation method of Schoch. The average molecular weight of the whole amylose component was found to be 70,000 ; this value was also obtained by calculation from the results obtained from each fraction. Meyer obtained 0·3 per cent. of tetramethyl methyl *D*-glucoside from the methanolysis products of the methylated maize amylose, whereas theoretically 0·2 per cent. would have been obtained if the amylose molecules were branched. If this result is correct, and the estimation of tetramethyl methyl *D*-glucoside to within 0·1 per cent. is difficult, it would indicate slight branching in some, but not all, of the amylose chains. Similar results have been obtained with potato amylose, although the fraction of high molecular weight contained only 700 glucose residues. Potter and Hassid[76] have prepared a variety of amyloses from different botanical species and investigated their molecular weights under the mildest conditions available. The values obtained from osmotic pressure measurements of the acetylated amyloses in chloroform varied from 100,000 to 210,000 (see Table IV), indicating that the molecular size of amylose may vary according to the botanical source.

Amylose has a very small reducing power towards alkaline copper reagents.[78] Meyer and his co-workers[77] have used the dinitro salicylic acid reagent of Sumner to determine the 'reducing' end groups present in amylose, thus affording an estimate of the molecular size. Since the values obtained correspond to those obtained by osmotic pressure and viscosity measurements,

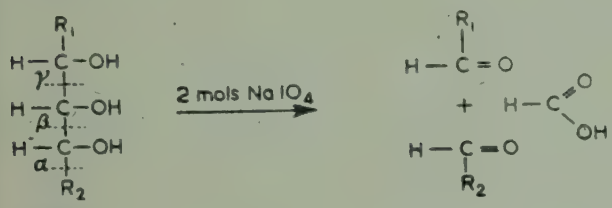
it would appear that one end of the chain of glucose residues is terminated by a reducing glucose molecule.

Hanes[79] and Freudenberg[80] proposed a screw-like structure for amylose (XX). This view has received support from French and Rundle[81] and from Dombrow and Beckmann[82] as a result of X-ray diffraction studies of the solid amylose-iodine complex and of amylose solutions respectively. The results obtained indicate that amylose consists of long chains of glucose units arranged in the form of helices, each turn of the helix consisting of six glucose residues and that in the amylose-iodine complex, an iodine atom is located inside the turn of each helix. The dichroism of flow exhibited by a solution of the complex provided confirmation of these results.[83] Rundle and his co-workers[61, 81, 83, 84] have developed the theory that when complexes are formed between amylose and such agents as iodine, alcohols and fatty acids, the long linear starch molecules form helices around the complexing agents. They propose that the stability of the complex is the result of dipolar action. Katybeck and Kerr[85] have shown that the phenomenon is not due to adsorption.

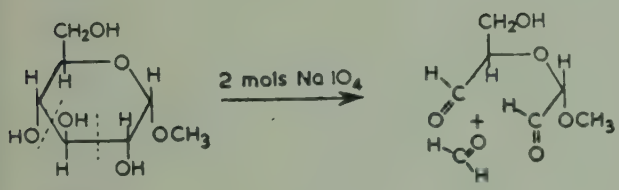
Within recent years, Hirst and his collaborators[86] have devised the first practical alternative to the methylation procedure for structural investigations of the carbohydrate group, and in particular of amylose and amylopectin. The method is dependent upon the observation of Malaprade[87] that a molecule containing an α-β-diol system (XXI) is oxidised quantitatively by sodium *meta*-periodate with cleavage of the carbon-carbon bond, into two aldehydic groups. A molecule containing an α-β-γ-triol grouping (XXII) will reduce two equivalents of sodium periodate with the simultaneous production of two aldehydic groups and one molecule of *formic acid*, which may be estimated quantitatively. Jackson and Hudson,[88] for example, have utilised the method to show that α-methyl *D*-glucoside (XXIII) contains three adjacent hydroxyl groups and hence to prove the presence of a pyranose ring in the methyl *D*-glucoside. Halsall, Hirst and Jones[89] have pointed out that the polysaccharides show a marked tendency to undergo over-oxidation in the presence of an excess of periodate, resulting in the production of more than the theoretical quantity of formic acid. Using potassium *meta*-periodate, which is only slightly soluble in water, and maintaining a low concentration of formic acid, they have observed that the theoretical quantity of formic acid is liberated from the end groups in starch, cellulose, maltose, methyl maltoside and various other polysaccharides, in about 150 hours at 15°. On oxidation with periodate β-methyl maltoside (XXIV) yields one



(XXI)



(XXII)



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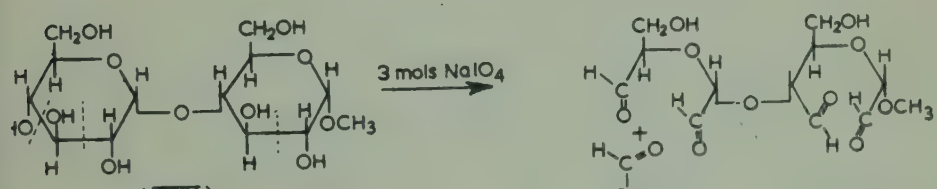

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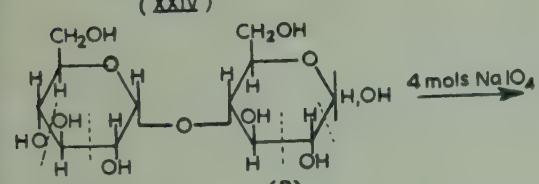

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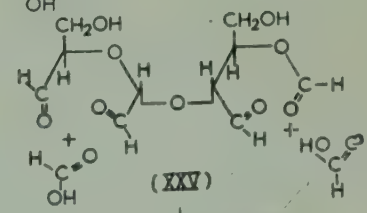
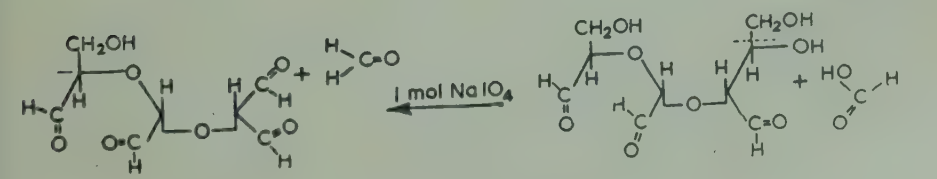


(XXV)

Hydrolysis



molecule of formic acid, while maltose (II) yields three molecules of formic acid, one from the non-reducing glucose residue (A) and two from the reducing glucose residue (B), as a result of the hydrolysis of the formic ester (XXV). Similarly, if the amylose molecule is unbranched, thereby consisting of a terminated chain of glucopyranose units joined by α-1 : 4-linkages (XXVI), it should yield three molecules of formic acid on periodate oxidation. On this assumption, Halsall, Hirst and Jones[89] and Potter and Hassid[76] have determined the average chain length of amylose and obtained figures varying between 400 and 1,000 (Table IV), which are in good agreement with those obtained previously by the methylation method of end-group assay. The latter investigators have found that the oxidation may be carried out more rapidly with sodium *meta*-periodate at 2°, a quantitative yield of formic acid being obtained after 25 hours. The periodate oxidation method possesses many obvious advantages over the methylation method, which is time consuming and tedious. The utility of the method is illustrated by the fact that an end-group determination by the periodate oxidation method may be carried out on as little as 0·2 gm. of polysaccharide. Meyer and Rathgeb[90] have described a further modification of the periodate oxidation method of Halsall, Hirst and Jones. They use a solution of sodium *meta*-periodate and carry out the oxidation at 0° at a *p*H between 4·2 and 5·8. The quantitative yield of formic acid is obtained after 20-30 hours. Under these conditions of controlled *p*H, the formic esters arising from the reducing end-groups of the polysaccharides, are not hydrolysed and consequently maltose and similarly an unbranched amylose molecule, will give only two molecules of formic acid.

The extensive data obtained by Potter and Hassid[76] indicates that there is little or no branching in amylose, since in the case of potato and Easter lily amyloses, there is good agreement between the molecular weights determined by osmotic pressure measurements and by the periodate end-group method and with tapioca, wheat, corn and sago amyloses, the difference corresponds to no more than two or three branching points in the amylose molecule. Higginbotham[93] has found that gels of different amyloses possess different strengths and stabilities, and he suggests that the most reasonable explanation of these facts is a different, but slight degree of branching in each amylose. These differences may be due, however, to mixtures of varying proportions of low and high polymeric homologues.

Schoch and his co-workers[53] have devised a technique for the fractionation of amylose by succe sive partial precipitations with

TABLE IV

The Molecular Weight and Average Chain Length of Amylose from a variety of Sources

Source	*Molecular Weight*	*Average number of glucose residues per molecule, calculated from the molecular weight*	*Average number of glucose residues per non-reducing end-group from periodate oxidation*	*Ref.*
Tapioca .	210,000	1,300	980	76
Potato .	150,000	930	980	,,
Wheat .	140,000	860	540	,,
Corn .	130,000	800	490	,,
Sago .	120,000	740	420	,,
Easter Lily	100,000	620	640	,,
Corn .	78,000	—	—	91
Potato .	—	—	500	30
Potato .	82,000 to 130,000		—	92
Wheat .	90,000 to 156,000		—	,,
Maize .	40,000 / 340,000 } 70,000	200 / 2,100 } 430	—	77
Apple .		560	530	92

n-octyl alcohol and from an investigation of the properties of these fractions it would appear that amylose consists of a continuous series of homologous linear polymers rather than a limited number of discrete components (see Chapter 6).

In conclusion, it would appear that on the evidence presented here, the amylose moiety of different starches is very similar, but not identical, the differences being due to a slight degree of branching or to a mixture of polymeric homologues or both.

TABLE V

A Comparison of the Properties of Amylose and Amylopectin

	Amylose	*Amylopectin*
(1) Reaction with iodine . .	intense blue	red-violet
(2) Blue value[35]	1·35	0·05
(3) Reaction with amorphous β-amylase preparation . . .	95-100% conversion to maltose	50% conversion to maltose
(4) Molecular weight . . .	40,000-340,000	4,000,000-6,000,000
(5) Average number of glucose residues per non-reducing end group	200-2,100	20-25
(6) X-ray analysis	High degree of crystallinity	amorphous
(7) Solubility in water . . .	sparingly soluble	very soluble
(8) Stability in aqueous solution .	retrogrades	stable
(9) Properties of the acetylated derivative	fibrous, forms tough films and fibre	amorph powde brit

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Amylopectin.—A knowledge of the properties of the amylose component of various starches and also of the whole starches described previously, permits many of the general properties of the amylopectin components to be predicted.

Since the methylated cereal and root starches, on methanolysis, yield some 4 per cent. of 2 : 3 : 4 : 6-tetramethyl methyl glucoside and methylated amylose gives very small amounts of end group, it is to be expected that the majority of the terminal glucose residues will be encountered in the amylopectin fraction of starch. Experimentally, it is found that amylopectin contains some 4 to 5 per cent. of end group (see Table V) from which it may be deduced there is one terminal glucose residue in amylopectin for every 20 to 25 glucose residues. These figures correspond to a molecular weight of about 4,000 which is much lower than the value obtained by physical measurements, which indicate values from 12 to 240 times this value.[38, 76] Amylopectin is practically without reducing power and it is evident that the polysaccharide contains less reducing glucose residues than terminal non-reducing sugar units (end groups). Furthermore, amylose gives little or no dimethyl glucose, and it is inferred, therefore, that amylopectin consists of chains of glucose residues joined through α-1 : 4-glucosidic links in such a manner that some 4 to 4·5 per cent. are end groups and that a similar percentage are linked in addition through the hydroxyl group on C_2, C_3 or C_6. The structure of amylopectin is, therefore, similar to the apparent structure suggested for whole starch, prior to the identification of two chemically distinct components; the simplest interpretation of the facts is, therefore, accommodated by the Haworth-Hirst laminated structure (XVIII). Exhaustive methylation of amylopectin with caustic soda and methyl sulphate results in a methylated amylopectin, which is a multi-branched polymer of high molecular weight and which yields, for every end group which produces tetramethyl glucose, a molecule of dimethyl glucose from the point of branching in the polysaccharide chain.[36] It has been demonstrated[36, 37] that the dimethyl glucoses contain 2 : 3-dimethyl methyl glucoside and that it arises directly from the starch and is not an artefact.[34]

After the removal of the amylose, as a complex, amylopectin may be precipitated from solution by ethanol. The product deghus obtained usually contains traces of amylose, and further due. rification is necessary to obtain pure amylopectin. It is polymerized as a white powder, freely soluble in water with the forma- Schoch opalescent solutions resembling those of glycogen. Frac- fractionation ipitation of amylopectin, as its acetyl or methyl deriva-

tive, gave a number of fractions showing different viscosities,[94] but this does not prove that this amylopectin is heterogeneous since the amylopectin may have been degraded during the methylation or acetylation procedure. To the contrary, Meyer and Gibbons[57] have fractionated potato amylopectin by electrodialysis in the presence of iodine, and observed that the fractions showed no difference in chemical properties. Brown, Halsall, Hirst and Jones have determined the average chain lengths of a variety of amylopectins by the periodate oxidation method, and in the majority of cases they have confirmed the results obtained by the methylation method.[30] In the case of a polysaccharide molecule which has no appreciable proportion of reducing end groups, any pyranose sugar residue, which is linked to other residues only through C_1 (and is therefore an end group) or only through C_1 and C_6 (main chain groups), will give rise to one molecule of formic acid. A rapid and convenient method is thus available for determining the average percentage of end group present in amylopectin. The amount of formic acid liberated by the long unbranched or slightly branched amylose, even after allowing for oxidation at the reducing end group, is small in comparison with that given by the numerous end groups present in amylopectin, and to a first approximation may be neglected. Since the percentage of amylose in the various starches can be estimated from the iodine binding power, by the method of Bates, Rundle and French,[58] the average length of the unit chains in the amylopectin, present in the respective starches, may be calculated (see Table VI). This approximation holds only if the amylose is present to a small extent (*ca.* 20 per cent.) in the starch.

The results in Table VI show that the average percentage of end group obtained both by the periodate oxidation method and by the methylation method are in good agreement with one another. Few, if any, glucose residues linked solely through carbon atoms 1 and 6 (XXVIII) can, therefore, be present in amylopectin and amylose. It is also clear that the amylopectin moiety of different starches is not always identical; in fact, of those studied hitherto, the average chain lengths vary between 20 and 27 glucose units, and, in addition, the molecular weights of the corresponding amylopectins, as determined by osmotic pressure measurements of the acetylated products, range from 1,000,000 to 6,000,000. These facts suggest that, like the amylose, amylopectins of different botanical source vary in both their molecular size and in the length of the chains of glucose units.

The identification of the linkage between the unit chains present in amylopectin has been the subject of much research. The

TABLE VI

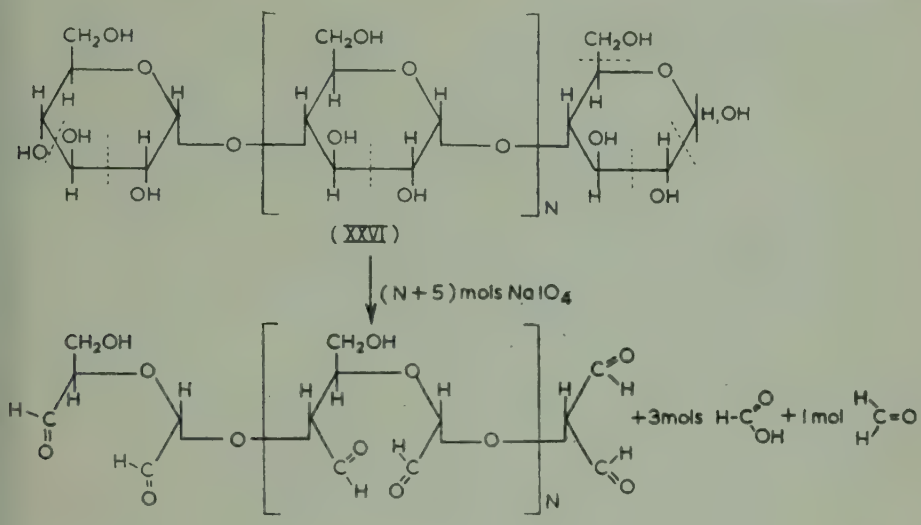
The Chain Length and Molecular Size of Various Amylopectins[30, 76]

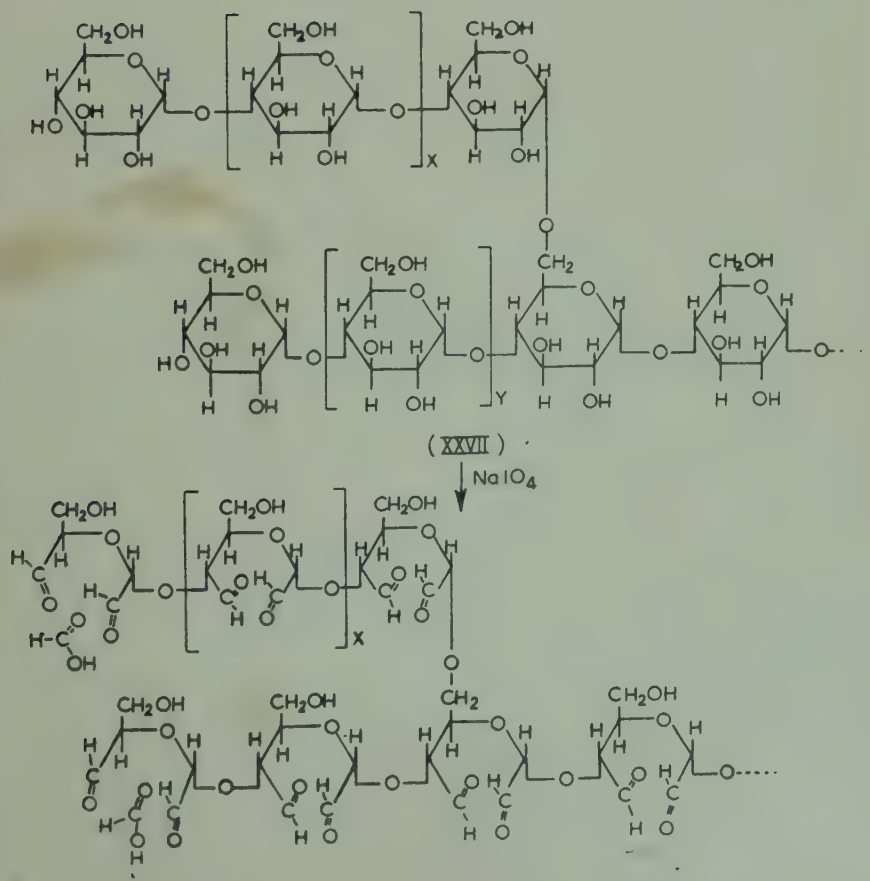
Source of starch	*Amylose content* %	*Average number of glucose units per non-reducing end groups*		*Calculated number of glucose units per non-reducing end group in the amylopectin fraction*
		(*a*) *periodate method*	(*b*) *methylation method*	
Arrowroot	20	31	—	25
Banana	21	27	26	21
Maize	23	25	—	20
Pearl manioc	16	24	—	20
Potato	18	31	25 ; 28	24 ; 26
Rice	14	23	—	20
Sago	26	24	—	18
Sweet Potato	18	32	28 ; 34	26
Tapioca	19	25	—	20
Wheat	19	26	—	21
Acorn	20	29	30	24

TABLE VI (*continued*)

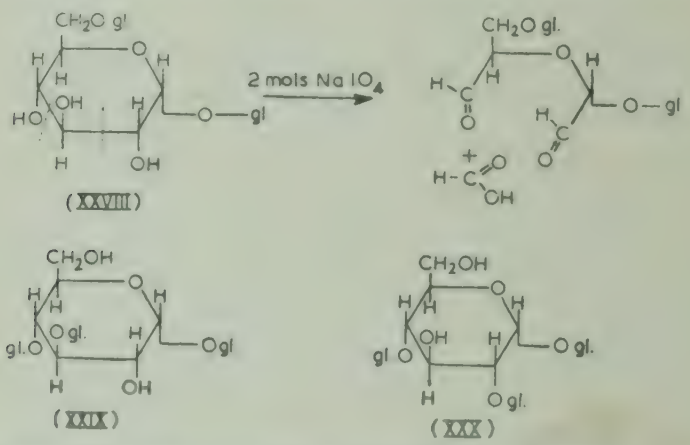
Source of amylopectin	*Average number of glucose residues per non-reducing end group by the periodate method*	*Molecular weight*[76] $\times 10^6$
Sago	22	1
Corn	25	5
Wheat	23	4
Easter Lily	27	3
Tapioca	23	3
Potato	27 ; 24	—
Waxy maize	20	—
Apple	24	—

isolation of 2 : 3-dimethyl *D*-glucose (XVII) from the hydrolysis products of methylated amylopectin suggests that some, at least, of the inter-unit bonds (branching points) are of the 1 : 6-type (XXVII). Uncertainty is caused, however, by the experimental difficulty encountered in the complete methylation of amylopectin and by the fact that trimethyl glucose tends to undergo some demethylation in methanolic hydrogen chloride. An alternative method of attack has been described by Halsall, Hirst, Jones and Roudier,[97] who have utilised the periodate oxidation method in a novel manner. An inspection of (XXVII) shows that when the side chain is attached to C_2, oxidation with periodate will disrupt the bond between C_3 and C_6, and after subsequent hydrolysis, this sugar residue will no longer be recoverable as glucose. On the


(XXVI)
(N + 5) mols NaIO4
+3mols
+1mol

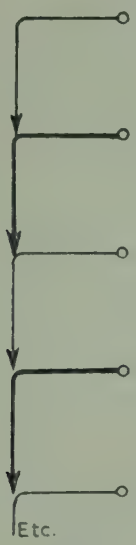

(XXVII)
NaIO4

other hand, if the side chain junction is at either C_3 or C_2 (XXIX) or (XXX), there will be no α-β-diol grouping, and therefore no attack by periodate. In these circumstances, the glucose residue will be recoverable as glucose after hydrolysis. Various starches, namely, potato, acorn, sago and waxy maize starch were oxidised with periodate and the oxidised material hydrolysed. The resultant products were then examined by the method of paper partition chromatography as described by Partridge[98] for the micro-separation and detection of the reducing sugars. In every case, glucose was detectable. An estimate of the amount of glucose produced by the separation of the glucose on filter paper by partition chromatography, followed by extraction of the

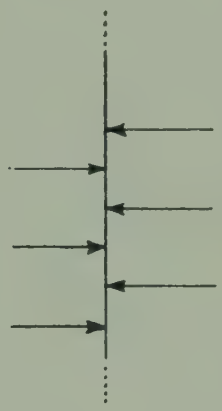


glucose from the paper and a subsequent estimation by the use of Somogyi's micro-copper reagent,[99] revealed that less than 1·3 per cent. of glucose, by weight of the starch used, was obtained. It is clear that in all these amylopectins, more than 75 per cent. of the inter-unit bonds (side chain linkages) are of the 1 : 6-type and, therefore, only a very small proportion of them can involve the hydroxyl groups on C_3 and C_2 (see also G. C. Gibbons and R. A. Boisonnas[113]).

The chemical results outlined above are most simply explained by the laminated formula, first suggested for glycogen and starch by Haworth, Hirst and Isherwood.[66] This formulation (XVIII) is purely diagrammatic and was not intended to suggest that there is any degree of symmetry in the amylopectin molecule. This structure may be written in the form of (XXXI). It is then clear that in this suggested structure there is present a combination of glucopyranose residues (represented by the vertical line in (XXXI)), which are linked by 1 : 4-junctions to the extent of about 96 per cent., the remaining 4 per cent. of linkages, repre-

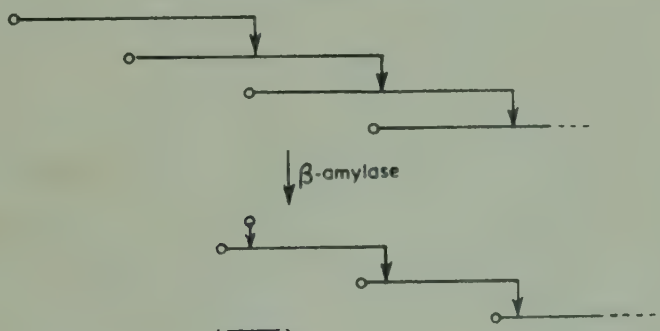


(XXXI)



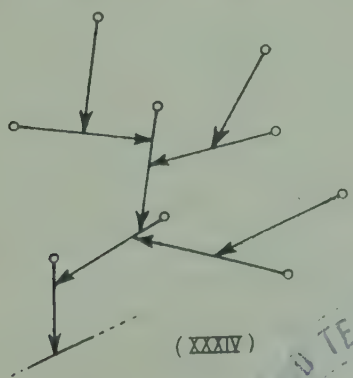
(XXXII)

Branched chains of ca. 20 1 : 4-linked glucose residues are attached to alternate glucose units in the main chain (vertical line). The junction is to C_3 or C_6 of the glucose units.



(XXXIII)

The amylolytic degradation of amylopectin as represented on the Haworth Hirst laminated formulation.



(XXXIV)

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sented by the arrowheads, being largely of the 1 : 6-type. The side chains are linked to the main chain by the 1 : 4-type of junction only. This formula differs from that of Staudinger and Husemann[100] (XXXII) in which the main chain is pictured as of the amylose type, consisting of glucopyranose residues linked through 1 : 4-linkages *only*, the branch chains being attached to the main chain by 1 : 6- and 1 : 3-linkages. Whilst the laminated structure of Haworth and Hirst explains the known facts relating to the chemical constitution of amylopectin, investigation has not yet been able to show at what point the chain branching occurs (i.e. the position of the arrowheads in the formulation (XVIII)). The mode of action of the amylases has been used as evidence for the structure of amylopectin, but in view of the lack of reliable knowledge of the specificity of the amylases, the evidence is unreliable. The position is rather that a consideration of the chemical evidence, in conjunction with the established facts of amylase action, results in a greater understanding of the starch problem. For example, β-amylase degrades amylopectin to maltose, to the extent of some 50 per cent., the residual 50 per cent. remaining as a limit-dextrin, termed α-amylodextrin, which is resistant to the action of β-amylase.[95] The β-amylase is capable of hydrolysing 1 : 4-α-linkages with the removal of maltose, and complete conversion to maltose does not occur, owing to the presence of linkages other than of the 1 : 4-type occurring in the molecule.[96] Since chemical evidence has shown that these linkages are, in the main, of the 1 : 6-type, it is assumed that the amylolysis ceases when the 1 : 6-linkages, or branching points, are reached. An examination of the limit-dextrin by the methylation procedure has shown that it contains an average of one glucose end group per twelve glucose residues.[95, 96] Oxidation of the dextrin with potassium periodate produces an amount of formic acid corresponding to eleven glucose residues per non-reducing end group.[95] Since the limit-dextrin has end groups, it follows that enzymic hydrolysis ceases at some point *as yet undetermined* before the 1 : 6-linkages, or branching point is reached (see XXXIII). Furthermore, the evidence reveals that the limit dextrin has an *average* chain length which is half of the original amylopectin, hence on the average, the branching points would appear to be half-way along each unit chain. However, end-group determinations only give an average figure; consequently, the branching points may be situated half-way along each unit chain, thus giving a symmetrical molecule, or alternatively, they may be distributed at random, since both possibilities could give the same result.

Meyer[101] has proposed a multi-branched structure (XXXIII) for amylopectin in order to explain the results he obtained by amylolytic degradation. He pictures the macro-molecule as consisting of a multi-branched chain, the branches of which are linked to the main chains by α-1 : 6-linkages only, the main difference between this structure and the laminated formulation being the possibility of more than one branch in attachment to any given unit chain. As mentioned previously, end-group determinations only give an average figure, depending upon the relative proportions of tetramethyl to trimethyl glucose, and consequently a decision between the multi-branched structure of Meyer and the laminated formulation of Haworth and Hirst cannot be made from this line of inquiry, since both would give the same result. However, the results of the disaggregation of methylated amylopectin to material of lower molecular weight, but containing the same proportion of end groups[41, 46] are difficult to reconcile with the idea of an irregularly multi-branched structure for amylopectin. The laminated formulation for amylopectin was advanced as a first approximation only, in order to explain the known experimental facts, but it is not to be regarded as the final or complete structure of amylopectin. Pacsu and Hiller[102] have advocated a novel structure for amylopectin, based on the assumed occurrence of a small proportion of non-cyclic hemi-acetal linkages (cf. also Dumazert[112]). However, this type of structure has been refuted by Halsall, Hirst and Jones,[30] on the basis of periodate oxidation experiments, and also by Bourne and Peat.[55]

An advance of the greatest significance in the chemistry of starch has been achieved by Bourne and Peat,[103] who have synthesised amylopectin by the agency of an enzyme, termed Q-enzyme, isolated from potato juice. This substance was observed to act on *D*-glucose 1-phosphate, in the presence of potato phosphorylase (P-enzyme), with the formation of a product possessing characteristic properties of amylopectin; the synthetic product gave a red colour with iodine, 46 per cent. conversion to maltose with β-amylase and an average chain length of 20 glucose units (see Chapter 2).

One of the most important problems still awaiting solution is whether in the native starch granules, there is some form of union between the amylose and amylopectin portions. A further question arises as to whether starches from different varieties of the same botanical species or starches from the same variety, vary during different parts of their life cycle in the proportions of amylose to amylopectin, or in the molecular size and average

chain lengths of the components. Meyer and Heinrich[104] have claimed that starches extracted respectively from potato tubers, leaves and shoots contain different proportions of amylose and amylopectin, the shoots in particular having a high proportion (about 46 per cent.) of the amylose component. Samples of starches, present in the tubers of two varieties of potato, namely Golden Wonder and King Edward, were examined by Halsall *et al.*[105] at intervals throughout the period of growth of the plants. No significant variation in the proportions of amylose and amylopectin was observed during growth and, furthermore, the amylopectin component of the two varieties of potato appeared to be identical.

A number of starches which behave in an anomalous manner have been described. A typical example is Floridean starch, which gives a deep violet to brown colour with iodine, resists attack by β-amylase and is structurally different from normal starches, in that it contains a large proportion of 1 : 3-linked glucose residues.[106] Sweet corn[35] is also interesting, since it contains both starch and a polyglucose that resembles glycogen (animal starch) in that it gives a red colour with iodine, and possesses a similar percentage of end groups.

Many problems associated with the structure of the starch granule still await solution, but with the rapid advances in experimental techniques that have been reported within recent years, new fields of inquiry have been opened up and it is to be expected that far-reaching advances will be achieved in the near future.

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Chapter 4

SOME PHYSICAL PROPERTIES OF STARCH

Pure starch, as distinct from commercial starch, is a white, odourless, tasteless, neutral powder, insoluble in cold water or organic solvents. In the well-dried state it is hygroscopic ; when it is air-dried, starch contains quite appreciable amounts of water. The colour of commercial starches may vary from white to a shade of grey or brown, their reaction may be either alkaline or acid. The odour of a commercial starch is often characteristic ; it is especially noticeable when the starch is treated with hot water to form a paste. The densities of the more important starches are all approximately 1·625, although that of potato starch is somewhat higher.

The study of the physical properties of starch is a fascinating one, and leads to information explaining a number of peculiarities that may often be observed. Why, for example, should one batch of starch be more readily convertible to dextrin than another ? or give a stiffer paste than a previous delivery ? or require more heat to form a paste when treated with water ? Again, why should one paste give more adhesive or stable mucilages than another ? or take longer to reach the same viscosity when converted to dextrin by enzymes ? Physical chemistry often gives the answer to such questions.

Much of the older literature on starch was confused and even contradictory since it was not realised that in most starch systems one was dealing with a mixture of substances and not an individual substance. Now that chemically homogeneous starch fractions are available much work awaits to be done on the physical chemistry of the two components and the effect of various agents on their physical properties when alone or in admixture with each other.

Starch is insoluble in cold water but swells to some extent when placed in it, and shrinks to its original size when dried to its previous water-content. Starch containing 42 per cent. of water appears as a fluid, but the slightest pressure on it causes it to solidify and appear as a damp solid cake. The paste flows very slowly, but if attempts are made to pour it out of a container quickly it solidifies. By the action of heat on a starch suspension,

a stage may be reached which passes that of true swelling, and the starch gelatinises. The granules swell to many times their original size and some starches, e.g. potato starch, burst, releasing an amount of starch material from the inner portion of the granule which goes into solution. A starch paste as usually made therefore consists of granules in various stages, some partially swollen, and some burst, together with soluble material and débris of the outer portions of the burst granules. Starch pastes as usually prepared are therefore not homogeneous.

The actual structure of the granules of starch has received much attention. Nägeli[1] assumed that they are built up from rounded or oval micellar units arranged symmetrically. Later, A. Meyer[2, 3] postulated that the granules are built up of dendritic crystals of starch substance which are densely packed in concentric layers ; intermediate layers separate these and consist of the crystals in a less densely-packed form. The water held in the starch was considered by this worker to be distributed between the crystals in such a manner that the less densely-packed layers contained the greater proportion of it. The effect of density of packing and moisture-content on the appearance of the granules is held by Meyer to account for the striations which some starches show under the microscope.

Fifty per cent. of the starch granule dissolves away when treated with dilute sulphuric acid for several months. The remaining structure is as highly refracting as ever so that not only is little, if any, crystalline material leached out but it must be twice as birefringent as the average of the whole granule. This crystalline residue is amylodextrin produced by hydrolysis of the starch in the granule form and the spherocrystals resemble starch granules very closely. The X-ray diagrams are indistinguishable from those of undegraded starch. Thus the organised crystallite regions in the starch granules resist hydrolysis and the heterogeneous regions of amorphous material are susceptible to attack. Frey-Wyssling[35] suggests that the positive sign of birefringence, with respect to the radius of the granule, suggests a radial distribution of extended chains rather than coiled helices suggested by Hanes[8] and by Freudenberg.[9]

Katz[4] and Samec[5] have shown that the structure suggested by Nägeli is probably the correct one, and this view is supported by O. A. Sjostrom,[6] who has studied the microscopical appearance of starches, both untreated and of the 'soluble' type, and has obtained excellent photomicrographs of the various granules swelling in water. In some of the photomicrographs the micellar structure is clearly shown. A. E. Hanson and J. R. Katz[7] found

strong evidence in Lintnerised wheat starch of orderly concentric and radial arrangement of globular units of small dimensions, the concentric connections being stronger than the radial ones, as can be seen by an examination of Sjostrom's photographs. In aqueous glycerol the successive layers peel off like the layers of an onion, and in completely dextrinised starch the entire granule from the surface to the centre appears to be made up of these concentric layers, showing that the micellar arrangement is the same throughout the granule. The photograph by Radley and Young shows these layers quite plainly.* However, N. P. Badenhuizen[22] considers that the block structure described by the above workers is not preformed in starch grains, but arises during swelling and comes from homogeneously resistant layers of the granule. After swelling different starches with various agents, the individual blocks cannot be separated from each other by a micro-needle, but appear to be fastened together with a soft swollen substance. Badenhuizen acted on starch with chromic acid to make the blocks more apparent, and found them to be about 1 μ in size.[23] From all the evidence this worker considers that starch is a chemically homogeneous product, containing various impurities derived from the amyloplast or the factory which affects its colloid properties. The grain forms a transition between liquid crystals and gels with a sub-microscopic structure and he considers that there is no question of true crystalline particles (micelles) but that the molecules form a delicate, loosely bound network.

A. Frey-Wyssling[35] considers the double refraction shown by starch granules to be due to strains, possibly in a radial direction, and not to the structure of the starch. He points out that the double refraction can be altered by mechanical means and concludes from this that the granules do not possess a lattice of compact crystalline strands but a brittle molecular structure that can easily be destroyed.[83] The structure of modified starches has been examined by R. Haller[97] who, by treating them with gold chloride or ammoniacal silver salt solutions followed by copper-oxide-ethylenediamine solution, was able to follow the dissolution of the starch material leaving lamellar, concentric membranes behind where the gold or silver salts had collected.

H. Speich[89] has studied the structure of starch granules and determined the degree of swelling of potato starch grains that had been carefully dried over P_2O_5 when dispersed in 28 different liquids. Volume changes varied from 68·88 per cent. increase in glycol to 7·20 per cent. decrease in salicyaldehyde. For water

* Photomicrograph No. 1, Vol. II.

the increase was 57·99 per cent. In general the more hydrophilic the liquid the greater the swelling and the more lipophilic, the less the swelling of the starch grains. According to birefringence determinations in liquids of different refractive indices, potato starch grains behave toward water, water-alcohol mixtures, alcohols and aldehydes as permeable aggregates of small rods, while toward lipophilic (non-polar) liquids, they appear to possess homogeneous submicroscopic structures. Differences of birefringence can be demonstrated in different parts of the grains (·0134, whole grain ; ·0124, periphery). For a wave-length of 589 m. and temperature of 25° the refractive index is 1·535 in the direction of the optical axis. Relative to temperature effects on refraction, the behaviour of the starch grains is between that of solid and liquid bodies. X-ray diagrams of the carefully dried starch grains obtained by this worker showed no well-defined crystal lattice structure but a less well-organised molecular framework.

Starch granules are of various shapes, a spot, the hilum, situated centrically or eccentrically being observed with many starches and this may be accompanied in some variations of starch by concentric lines. These striations are best seen by oblique illumination and careful focusing and it may be necessary, in some starches, to stain the granule with dilute chromic acid to render them visible. From what has been said above it would appear that these striations are significant with respect to the organic structure of the granule. Kerr,[15] however, points out that when crushed a striated granule might tend to fracture first along parallel lines, but such fractures occur radially. These markings do not correspond to definite solar intervals in the growth period of the plant but they may represent periods of relative inactivity in the deposition of the starch material. If the outermost layer is deposited last, growth is by apposition, if it is deposited first, growth is by intussusception and it might be expected to be more dense, less hydrated and less susceptible to enzymic action which is found to be the case.

In this controversy several workers[21, 24–26] have presented the same general picture of the microscopic organisation of the starch granule. J. W. Evans[94] has presented photomicrographs of developing corn starch showing that, as the kernel becomes more mature the starch granules change from circular to polygonal in outline.

O. E. Stamberg[43] has calculated the surface area of seventeen types of wheat flour from the dimensions recorded by Grewe and Bailey.[44] The average is 2,004 sq. cm. per gm. and individual

samples range from 1,780 to 2,339 sq. cm. per gm. The granules below 7 μ in diameter constitute some 82 per cent. of the total number, but the bulk of the mass, some 93 per cent., is due to granules larger than 14·8 μ, which contribute some 76·4 per cent. of the total surface area. The surface area of potato, wheat, corn and rice starches are 853, 1,907, 3,077 and 8,000 sq. cm. per gm., respectively, giving a rough ratio of 1 : 2 : 3 : 8.

These surface areas and the average particle size are of importance industrially, e.g. in the manufacture of the so-called 'Baking Powders' (see Vol. II). The power of starch to adsorb various substances does not appear to depend on the surface area presented by the various starches (*vide infra*).

The particle size and structure of wheat starch has also been examined by L. H. Pulkki.[82]

The properties of different starches have been tabulated by S. M. Strepkov and C. K. Kuramschin[32] and other workers.[79] H. Rodewald[33] has studied the heat of wetting of starch, treating it from the thermodynamical viewpoint[34] and deriving a mathematical expression for the heat of wetting.[36] His work was followed by that of H. Gaudechon[42] who measured the heats of wetting of starch by sixteen different liquids and by A. I. Al'tov.[85] The latter found that the heat of wetting of starch by solutions decreased in the order: water, glycerol, glucose, sucrose. The heat of wetting by urea solutions increases with increase in the urea concentration and he obtained definite evidence that urea is adsorbed by starch (see p. 86).

The contact angle between various liquids and starch gels have been measured by P. A. Pchelin[160] whilst E. H. Zubaschenko[80] studied the effect of various compounds on the contact angle between their solutions and films of starch.

The specific heats of wheat starch samples containing from 0-33·6 per cent. moisture had been measured by Rodewald[73] who found that the value for specific heat varied, but not uniformly, from 0·2697 to 0·3054.

Nossian[78] examined seven different starches for their hygroscopicity and found that wheat starch was the most and acorn starch the least hygroscopic. The vapour tension and its connection with the bound water in starch has been studied by Z. P. Chesheva.[81]

Adsorption by Starch.—Starch which has been thoroughly washed with distilled water and then made into a suspension with a further quantity of water does not liberate an appreciable amount of electrolytes, which is in contrast to its behaviour in the dissolved state. Certain cations which are present in the

untreated starch are readily removable by a thorough washing with dilute hydrochloric acid followed by a thorough washing with water. According to G. Malfanito and A. N. Moschkoff,[119] when a potato-starch solution is frozen and allowed to thaw, the liquid separating contains most of the electrolyte present in the original starch; if the mass be well washed with water the ash of this portion consists almost entirely of phosphoric acid and they were able to reduce the ash-content of potato starch in this manner to less than 0·2 per cent.

A. Fernbach[130] early showed that the phosphorus in some starches could not be removed by washing with water or by treatment with dilute acids, and both Tryller[132] and Fouard[131] have shown that although the cations are removed by dilute acids, the latter have but little effect on the phosphorus-content. Starch solutions, after thorough dialysis, lose a portion of the phosphoric acid, and that the longer a starch solution is heated under pressure the greater is the amount of phosphoric acid which is lost upon dialysis.[19] The resulting increase in acidity and hydrolysis on heating is attributed by A. Tychowski and S. Mesior[133] to the liberation of phosphoric acid, which is almost complete after heating for 10 hours at 120° C. The phosphorus-content of canna and potato[15] starches are unaffected by acetylation, methylation or subsequent saponification. C. Tryller[132] has also shown that by washing with appropriate salt solutions and then with water until free from electrolyte the metallic atoms can be interchanged. It has been shown[135] that 90 per cent. of the phosphorus and 75 per cent. of the nitrogen in wheat starch can be removed by washing with alcoholic potash but potato starch retains its phosphorus when submitted to this treatment. Wheat starch pastes or wheat starch pre-treated with alkali is dephosphorylated by alcohol[135] but potato starch and intact wheat starch granules are unaffected.[136]

Dried, but not moist, starch granules adsorb from 5 to 6 times their volume of carbon dioxide, which is only incompletely removed in a vacuum although treatment with boiling water removes it completely.[45] T. J. Schoch[114] has pointed out that if dried starch is confined over various liquids the vapour adsorption is dependent on the hydrophilic nature of the liquid.

Cuprammonium solution swells starch granules[46] but they do not pass into colloidal solution, potato and wheat starches being affected differently by the cuprammonium solution.[47] The starches appear blue after treatment and contain some 12 per cent. of copper oxide. On washing with ammonia the copper content gradually decreases and the starch finally dissolves.

A. V. Rakovski[48] has studied this phenomenon and C. E. Guignet[49] notes that the ammonia present in the blue starch-copper compound is driven off at 40° C.

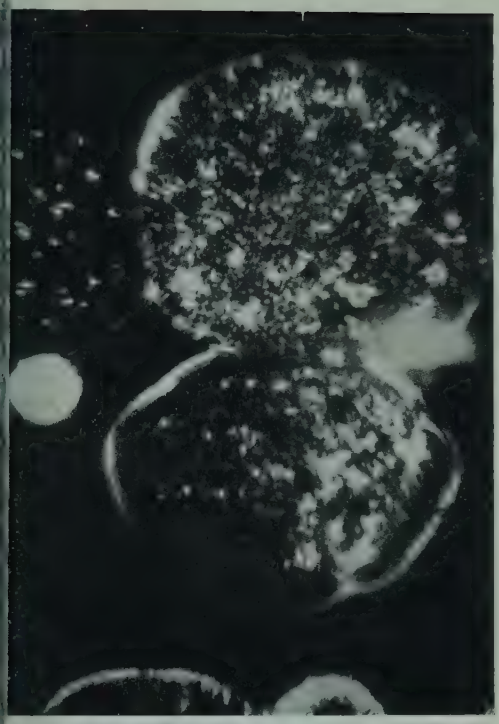
W. Traube[50] noted that solutions of alkali or ethylenediamine containing starch dissolve larger amounts of cupric hydroxide than when starch is absent. A cupra-ethylenediamine solution affects starch in a similar manner to that shown by cuprammonium solution. After repeated washing the blue ethylenediamine-copper-starch substance still contains nitrogen.

The adsorption of acids[51, 52] and bases[53, 54] by starch has been investigated by several workers, and it has been found[52] that adsorption is not a function of the granule surface per unit weight but that starches vary in their adsorption capacity. With hydrochloric acid the ordinary adsorption isotherm is followed for solutions up to 0·4 N with all the common starches except maize. The isotherm is also followed at ordinary temperatures using butyric and isovalerianic acids.[55]

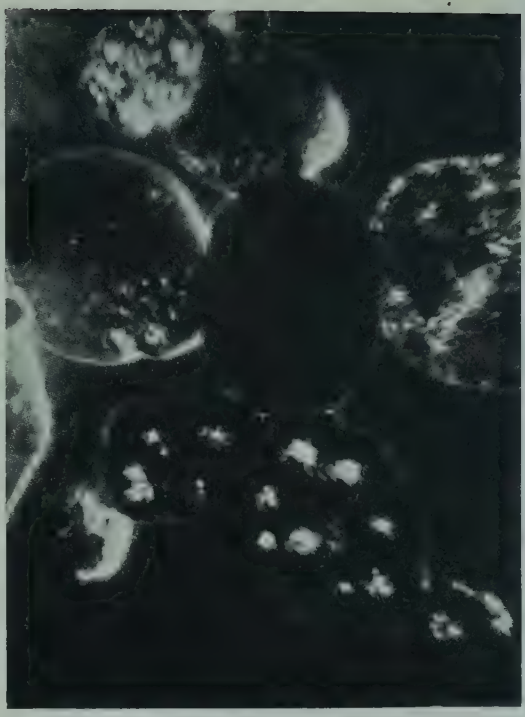
T. Pfeiffer and B. Tollens[56] have studied the sodium and potassium compounds of starch. E. Fouard[54, 57] found that the conductivity of solutions of ammonia and piperidine is diminished by the addition of starch and ascribes this to adsorption and not salt formation, which would increase the conductivity. A. Reychler,[58] however, considers that in these cases compounds analogous to the alcoholates are formed.

The adsorption by starch of barium sulphate,[59] alkaline earth and lead carbonates,[60, 61] sodium chloride and copper sulphate[62] has been examined by several workers. G. Carriere[63] finds that barium ions are adsorbed by potato starch or soluble starch suspensions but not by the colloidal solutions. A. V. Rakovski[64] considers that starch adsorbs little or no acids or salts from solution but that aqueous alkalis and hydroxides of heavy metals in aqueous ammoniacal solution are strongly adsorbed, and this worker has presented data on the adsorption of bases by starch.[65]

The author has treated starch in suspension with strong solutions of zinc, aluminium and magnesium sulphates, and after washing free from any trace of the salts has treated with acid and tested for the metals, using the fluorescence micro-tests described in the book by the author and J. Grant.[66] In each case the test showed the presence of the metal. The starch treated with one of the metallic salts mentioned, washed free from the salt and then immersed in a different salt solution showed, after washing free from the second salt solution, the presence of the metal of the second but not the first salt when treated with acid. The starch, therefore, appeared to behave like a zeolite, one metal



Photomicrograph No. 1.



Photomicrograph No. 2.

Photomicrograph No. 3.

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Photomicrograph No. 4.

os. 1 and 2. Spierer photographs of corn starch partially swollen in water at 70° C., showing luminous particles.

o. 3. Same as 1 and 2, but particles here are showing tendency to concentric arrangement.

o. 4. Acid-treated wheat starch, showing radial cracks and concentric arrangement.

(*Facing p.* 64.

Photomicrograph No. 5.

Photomicrograph No. 6.

Photomicrograph No. 7.

Photomicrograph No. 8.

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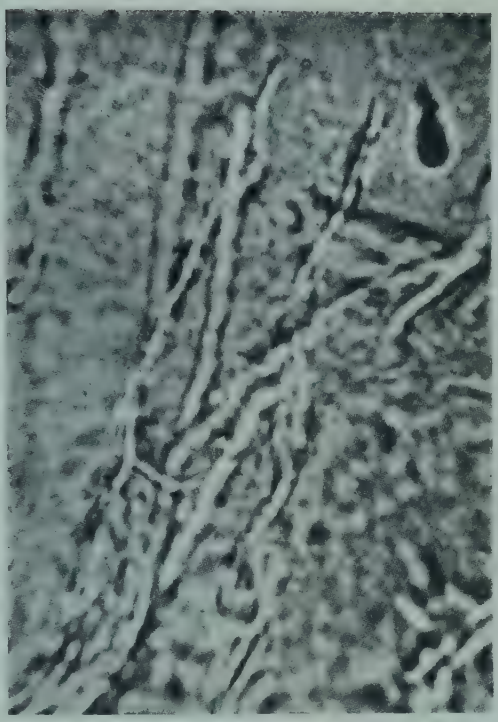
No. 5. Particles in active Brownian Movement on rim of partially swollen wheat-starch granule photographed with Spierer lens.

Nos. 6 and 7. Corn starch fully swollen at 95° C., then frozen, showing reticulation and Spierer lines.

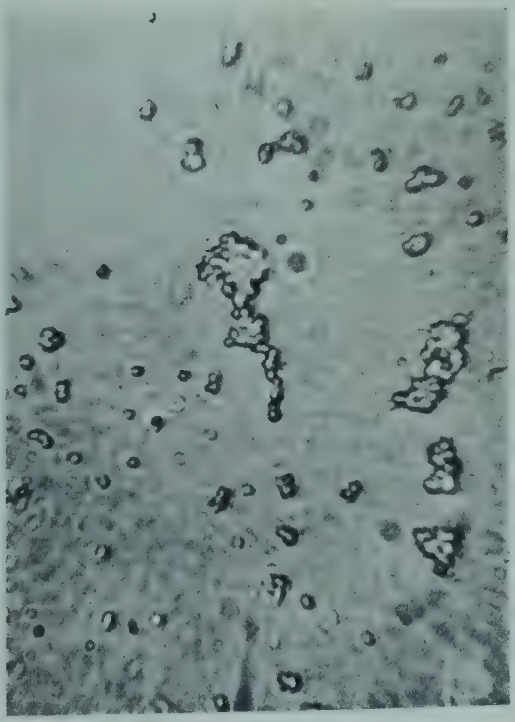
No. 8. Wheat starch heated at 70° C. in water then frozen. Spierer lines just beginning to form.

Photomicrograph No. 9.



Photomicrograph No. 10.

Photomicrograph No. 11.



Photomicrograph No. 12.

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ıs. 9 and 10. Same field of frozen wheat-starch gel; (9) with Spierer lens; (10) with ordinary lens.

ıs. 11 and 12. Dense luminous particles from starch taken with (11) Spierer lens and (12) with ordinary lens.

replacing another in the 'adsorption complex'. The amounts adsorbed were not large but gave very definite reactions.

Starch treated with a solution of a calcium salt adsorbs the calcium or, if treated with sodium chloride solution, the sodium. The 'sodium starch' swells much more than the 'calcium starch', showing that the metal present exerts a very definite effect. If oxidised starches are used the same differences in swelling power are shown. Now, potato starch contains phosphoric acid, the smaller granules containing a larger proportion of the acid and swelling more than the large granules. The phosphoric acid content therefore plays a part in the swelling behaviour of starch and thus has an influence on the viscosity.[70] By preparing samples of starch of approximately uniform granule size (e.g. by sedimentation) and forming the calcium, sodium, etc., starches from them, measuring the degree of gelatinisation in distilled water, the influence of the metal may be shown. A repeat experiment, but using a saline solution, would show the effect of chain-length on the swelling powers, as here the effect of metallic ions and of the phosphate radicle is eliminated.

The base exchange capacity is small and would appear to be in the neighbourhood of 3 milli-equivalents of metal per 100 gm. of starch.

Besides water, liquid ammonia, liquid hydrogen cyanide, formamide and formic acid are excellent gelatinising media for starch. It will be noted that all these liquids have a high dielectric constant, i.e. are capable of dissociating any hydrophilic cohesion between the starch molecules. The preferential adsorption of alkalis, e.g. sodium hydroxide, on to the OH groups of the starch (see p. 87) likewise brings about dissociation by neutralising inter-molecular attraction.[114] That the alkali is merely adsorbed is shown by the fact that it can be completely removed by repeated precipitation with methanol. If insufficient free water is present to hydrate the starch, as in saturated sodium hydroxide solutions, the starch does not gelatinise.

As will be discussed (see p. 87) gels may be obtained by the action of a Hofmeister series of salts but, as in the case of sodium hydroxide, the product does not approach the solution state and the internal granule structure is not completely disrupted. Peptising ions carrying a large inert residue, e.g. the quaternary bases, produce a much greater disruption and trimethyl benzyl ammonium hydroxide will give, in the cold, a limpid solution of low viscosity. It may be that the function of the quaternary ammonium ion is to act as a wedge and assist the prying apart of the molecules. Morpholine, trimethylamine and ethylene-

diamine act similarly in hot solution, in these instances apparently without the alkaline decomposition which is apparent on heating starch gels made with sodium hydroxide solution. In these cases it may be the hydrophilic amine group which associates with the starch by a process similar to hydration.

Films of Starch.—Hot solutions of starch or of amylose form strong insoluble films on the surface free to the air. Particulars of the extensibility and other physical properties of films made from various starches are given in Volume Two. Such films are readily removed from the surface of the paste and can be washed free of soluble components.[20] In the wet or dry state such films are strongly birefringent and are uniaxial negative with respect to normal of the film surface. Treatment with dilute iodine solution gives dark blue or black films which do not exhibit noticeable dichroism.

The starch chains in such films appear, to judge from the optical characteristics, to be grouped parallel to the film surface. The direction of most rapid increase in size of a crystalline mass is usually normal to the long axis of the molecule which, in the case of starch films, is normal to the surface of the film. With oriented films it is therefore possible to study the effect of swelling agents on the degree of swelling both laterally and normal to the starch chains. D. French has examined the swelling of oriented starch films[14] and finds they increase about 50 per cent. in surface area and some 300-500 per cent. in thickness. This would indicate that the amylose chain swells in a direction normal to the molecular axis without any decrease in length and as the amylose film is constrained by the molecular valency forces of the starch chains the greatest part of the dimensional change is in the direction normal to the film surface and normal to the molecular chains. In the starch granule such swelling, in a direction normal to the starch molecules, results in tangential expansion and a lowering of the interior pressure.

Another pointer to the configuration of amylose and amylopectin is given by the studies of R. L. Whistler and co-workers.[90, 91] It is known that linear molecules more readily form strong films than molecules which are less readily oriented due to their shape. The above workers find that amylose acetate forms tough pliable films whereas amylopectin acetate gives brittle films. Esters of the whole starch have been prepared by several workers (see Chapter 13) and several[92, 93] have noted the brittle character of the films which is probably due to the great preponderance of the non-linear acetylated amylopectin present.

Some Properties in Solution.—A. Coehn[120] appears to have

been the first worker to note that starch in solution or in aqueous suspension has a negative electric charge. Working with potato starch, F. Bottazzi and C. Victorow[121] noted that amylose shows no electrical migration, whereas under the influence of an electric current the amylopectin fraction is transported to the anode. F. Bottazzi[122] observed that in an acid solution the starch migrates towards the cathode, and in an alkaline solution towards the anode, no migration occurring in neutral solution. Z. Grużewska[123] also noted the anodic migration of the amylopectin fraction of potato starch, and E. Fouard[124] observed the phenomenon in ultra-filtered solutions, using the ultra-microscope.

Samec has employed[125–128] extensively the electrodialysis of starch solutions to effect a rapid separation of two fractions, one giving a jelly from which the clear mobile solution of the other is readily removed. By this method he obtained a fraction from potato starch, having 0·175 per cent. P_2O_5 and a soluble fraction quite devoid of phosphorus. Samec's method of preparing his starch solutions is to heat the starch and water under pressure to give a temperature of 120° C. for not more than three hours which gives a solution which separates sharply into two well-defined and readily separated layers. The lower, jelly-like layer obtained in this way contains about 7 per cent. of its weight of dry matter. This method[129] cannot be employed with soluble starches or starch derivatives which have smaller micelles, and to obtain a separation in such cases one must resort to precipitation with metallic salts and alcohol.

The Retrogradation of Starch.—J. Schoch[137] has recently applied the term ' retrogradation ' to all cases where starch shows a tendency to revert to a less soluble form, including such processes as the precipitation of starch from solution by alcohol, or by removal of a peptising electrolyte where the starch is dissolved in it, the insolubilisation of starch on freezing, or drying, as in the skin formation on pastes, and its deposition from aseptic solutions on keeping. Hirst and co-workers[138] have applied it to insolubilisation of starch on drying, and S. Woodruff and co-workers,[139–142] and Tanner and Inglis[158] have applied it to the changes occurring on freezing or alcoholic precipitation. Lampitt, Fuller and Goldenberg[143] have, with some justice, restricted the use of the term to those physico-chemical changes of state taking place in solutions, gels and pastes of hydrophilic colloids on ageing. In the following pages and especially in Chapter 8, by R. L. Whistler, there are some very definite distinctions in some of the properties of, for example, starch separated by freezing and that deposited on ageing.

L. Maquenne[159] noted that homogeneous starch pastes deposited solid matter on standing which is not coloured by iodine, or readily attacked by malt extract,[144, 146] and which, although soluble in caustic potash solutions, is but slowly acted upon by dilute mineral acids. The substance is deposited more readily at lower temperatures,[144–146] and its formation is favoured by the presence of mineral acids, whilst the amount and speed of the retrogradation is dependent on the concentration of the paste or solution.[145, 146] The deposited substance is not soluble in water at ordinary temperatures, and requires to be heated under pressure before solution is again obtained. In this case it is slightly soluble at 120° C., but has to be heated to about 150-155° C. before it is completely dissolved.[144]

Schoch (see Chapter 6) finds that starches which have been modified by oxidation, dextrinisation or ethylation show little tendency to revert, but in each case the modification of the starch must have passed some sort of ' diffuse limit ', as a lightly oxidised or dextrinised starch will retrograde. Several workers find amyloamylose to retrograde rapidly, and the ability of a dextrin to retrograde appears to depend on the molecular weight, the lower this value the less the tendency to retrograde.[147, 148] Other workers claim that this also applies to starch. Starch pastes of low concentrations behave similarly to starch solutions, but at higher concentrations they set to a gel on cooling and later show signs of syneresis. At still higher concentrations syneresis is absent.[144] The X-ray patterns of starch, ' soluble ' starch and dextrin solutions and pastes, as well as that of bread, are all sharper in definition after ageing and retrogradation,[149–151] but this is not observed with glycogen or non-retrograding dextrins.[152]

By the action of malt diastase on starch pastes maltose is obtained corresponding in amount to about 80 per cent. of the starch, and Maquenne and Roux further observed that on allowing malt extract to stand,[153] or by adjustment of the *pH* value with acid,[154] a larger amount of maltose was obtained, and they considered the amylopectin was a maltosan-like amylose.[155]

Using starch pastes containing about 10-20 per cent. of starch, or solutions of certain dextrins containing about 40 per cent. of solid matter, retrogradation may be noted after about 48 hours have passed, whereas a paste made by heating starch with an equal weight of water at 100° C. for 1 hour can readily be broken off short if allowed to stand for the same time at normal temperatures. From this J. R. Katz[156, 157] concludes that the swelling and stiffening of starch in water is an equilibrium process, and that the limiting condition thus depends on the temperature and

the relative amount of water present. This phenomenon is not characteristic for one variety of starch, but is shown by potato, rye, wheat, barley, maize, and other starches.

'Retrogradation' of Starch by Freezing.—S. Woodruff and her co-workers have studied the effect of freezing on the retrogradation of starch gels and have obtained a number of interesting results. Markedly different results were produced by freezing at — 2° C. and freezing at — 70° C., the gel in the latter case on thawing retained most of the slimy consistency of a newly gelatinised starch, and had a 'brittle' microscopic appearance, whereas the former had a spongy, fibrous structure showing heavy strands under the microscope. It was found that there was a transition temperature at — 25° C. below which the gel after thawing had the 'brittle' microscopic appearance, whereas the coarser microscopical appearance was always obtained by freezing above this temperature.

When material frozen at temperatures higher than — 25° C. was examined microscopically between crossed Nicols, strands of anisotropic material which had not existed before freezing could be seen, and when the granules were not completely gelatinised before freezing small anisotropic particles were seen which tend to form chains during freezing. When more completely gelatinised paste is used the phenomenon is not readily obtainable.

Gels 'retrograded' by precipitation with alcohol differed markedly from those 'retrograded' by freezing, in that between crossed Nicols they were luminous throughout and granules in the gels appeared to have been dehydrated but not re-oriented into strands, as by freezing. Re-gelatinising this substance gives a gel very similar to the original.

S. Woodruff[139] has also used the Spierer lens in this work, and the strands of reticulated, anisotropic material appear with even greater distinctness. For convenience she has termed these 'Spierer lines'. They are parallel, somewhat discontinuous, alternating bright and dark bands which follow the strand in the frozen structure. Amylose which has been allowed to retrograde for some months to a white flocculent material showed a 'brush-heap' or 'fish-egg' structure, but after dissolving at 95° C. and freezing, the material precipitated was fibrous, having similar Spierer lines and strands as the frozen starch gels. Amylose and amylopectin are indistinguishable by the reticulation shown on freezing. The photomicrographs 1-12, taken by Professor S. Woodruff, illustrate some of the results obtained (see p. 64).

Small rounded particles were observed in slightly swollen starch granules or in the fluid surrounding them. They were

separated and many showed anisotropy. Woodruff suggests that these particles may lose water of hydration during freezing and re-orient themselves to give the luminous Spierer lines, but her experimental work directed to proving this has, so far, proved unsuccessful.

Other Properties of Starch.—The birefringence of starch has already been mentioned. V. I. Nasarov[67] has noted that a suspension of potato starch in benzene displays, by side illumination, different colours according to its moisture-content; with 16 per cent. moisture it appears reddish-yellow, with 21 per cent. reddish-violet, and with 24 per cent. violet, the colours changing when the solution is warmed. It would be interesting to see if a rapid approximation of the moisture-content could be obtained by this method, using the Guild Colorimeter. B. Hošpes and B. Dmitrijev[68] have used a Lange's photo-colorimeter to characterise starch samples according to the granule sizes present. They find that the maximum light absorption by the starch-milk is dependent on the concentration of the suspended dry starch. The values with starches of small granule size are more than those of large granules. The rate of decrease in absorption by a suspension on keeping depends on the granule size, so that periodic observation of, e.g. 10 gm. starch in 100 ml. of liquid readily gives a means of characterising the starch.

A. M. Shkodin[69] has determined the distribution curves for water suspensions of four different grades of potato starch and showed that the size of most of the granules lies between 40 and 80 μ. The velocity of sedimentation of starch increases with increase in the concentration of the electrolyte present. Hydrochloric acid, sodium and calcium chlorides, lactic acid and calcium lactate were used, the last three at concentrations up to 5 mol per l. In the presence of ferric chloride the velocity of sedimentation reached a sharp maximum at 0·2 equiv. per l. Electro-ösmotic measurements showed a decrease of ζ potential in the presence of electrolytes. The effect of

$$Na^{+} < H^{+} < Ca^{++} < Fe^{+++} < Th^{++++}.$$

The maximum of the velocity of sedimentation in the presence of ferric chloride corresponded to the isoelectric point of the starch on the ζ potential curve. In the presence of electrolytes the starch precipitated in the form of aggregates containing 2 to 3 or more granules. The sedimentation rate of potato starch suspensions, containing 2-3 per cent. of starch, has also been studied by V. A. Smirnov[56] who used a photoelectric cell. He found the highest rate was at p_H 2 where the deposition was

A-type.

X-ray photograph of rice starch.

(Photographs by W. T. Astbury and reproduced by courtesy of 'Nature'.

B-type.

X-ray photograph of potato starch.

FIG. 4.1.

(Facing p. 71.

four times as fast as that at *p*H 6. From the point of view of starch manufacture, however, the best *p*H is at 4·5 for at this point the potato proteins are not coagulated and the settled starch is not contaminated with protein or acid. J. M. Chaves and S. A. Atuch[72] find that manioc starch precipitates fastest at *p*H 2·82, the sedimentation rate decreasing rapidly above this value.

The protective action of dextrin and starch on gold sols and Congo blue sols is inferior to that of gum-arabic.[71]

X-Ray Examination of Starch.—M. W. Beijerinck[27] in 1915 drew attention to the fact that, under certain conditions, deposits from cooled solutions of certain starches are crystalline and R. O. Herzog and W. Jancke[28] noted the crystalline form of starch. These workers also noted that amylodextrin is crystalline and starch acetate is amorphous.[29]

In order to obtain more information on the internal arrangement of starches and starch products a number of workers[98–102, 115] have examined starch by means of X-rays. The diagrams yielded in these investigations may be either an amorphous halo or show a few interference rings which show there is some crystalline or micellar structure prevailing in the sample.

In native starch granules two main types of crystal structure have been found, the tuber starches giving the so-called 'B' pattern and the cereal starches the so-called 'A' pattern. Intermediate types between these two patterns are called the C-types.[10] On precipitating starch pastes with various agents such as various alcohols, etc., the precipitated starch exhibits still another pattern, the *Verkleisterungspektrum*, paste pattern of 'V' pattern.

Whether a starch paste from any source gives the A, B or C pattern when crystallised from a starch paste appears to depend on the temperature of preparation.[37] Precipitation of insoluble starch by freezing or retrogradation or by evaporation of starch pastes at room temperature generally favours the formation of the crystalline material giving the B pattern. At temperatures somewhat above room temperatures the C-type is favoured and at 80-90° C. the A-type. The amorphous type is usually obtained at higher temperatures. The crystal unit cell parameters of the A- and B-type have been found by Bear and French.[11]

The diagrams opposite illustrate the A- and B-types whilst the C-type appears to be almost certainly a mixture of A- and B-types. The V-type is given by samples obtained by alcoholic precipitation from solution.[87] Such material is precipitated in a coarse and shapeless form when ethanol or propanol are added to starch solutions. The V pattern consists of three, or four, more or less sharp lines. The V pattern is simpler than the A or B patterns,

thus the molecular arrangement in this form is simpler than in the other types. Butanol or amyl alcohol give a fine precipitate of starch with a relatively rich X-ray pattern which, however, transforms to the V pattern on drying.[13] Tertiary butyl alcohol also gives a precipitate which gives a V pattern but with somewhat smaller ring diameter rings indicating a larger crystal lattice. Starch iodide also gives a fairly good V-type of pattern.[12]

Freshly precipitated starch of the V-type, if allowed to stand in a moist condition retrogrades to the B-type. At first it is readily dispersible, often in cold water, but this property is gradually lost. It also possesses the striking property of being able to absorb large amounts of iodine vapour even when quite dry.[14] W. T. Astbury, F. O. Bell and C. S. Hanes[115] found that a specimen of amylo-amylase precipitated by alcohol after electrophoretic separation gave a V-type photograph but using the synthetic starch prepared by Hanes precipitation with alcohol gave the B-type of photograph. It certainly appears that the phosphorylase present in the plant itself may have some influence on the manner of deposition of the starch and hence its X-ray diagram, but the above workers are more inclined to the tentative view that the conditions of deposition may be the deciding factor, and this view is also supported by R. S. Bear and C. F. Cori.[116]

R. S. Bear and C. F. Cori[116] prepared synthetic polysaccharides with muscle, heart and liver phosphorylase.[118] The polysaccharide obtained using muscle phosphorylase was able to exhibit both the B- and V-types of X-ray diffraction pattern. It had a blue iodine reaction and retrograded readily and was thus similar to plant starches. The similarities in the X-ray diagrams of plant starches and this synthetic polysaccharide included ring positions and relative intensities of the rings and also the alterations brought about in the intensities of the rings by wetting or drying. The polysaccharides formed with liver and heart phosphorylases[117] behave like glycogen in a diffuse pattern characteristic of an amorphous material having a red-brown iodine reaction and not retrograding. The interesting problem now to be settled is why muscle phosphorylase produces starch *in vitro* and glycogen *in vivo* and why it differs in this respect from liver, heart and brain phosphorylase which produce glycogen both *in vitro* and *in vivo* (see Chapter 2).

Some workers[15] consider that the A- and B-types have an extended molecular configuration and that starch of the V-type has a helical configuration. Thus Rundle and Daasch,[88] working with amylose plasticised by glycerol find the fibre spacing to be

7·5 Å, the ' B ' modification giving a spacing of 10·6 Å. The greatest polarisability of the glycerol-amylose fibre is parallel to the fibre axis and the chains are probably extended linearly.

Lampitt and co-workers[103] find one interference line present in both retrograded and non-retrograded wheat starch fractions prepared by milling (see below), and consider that the fundamental structural unit postulated to give rise to this line is present in both retrograded and non-retrograded portions. J. R. Katz,[37–41] who with co-workers distinguishes between a crystalline and an amorphous diagram and has shown how various treatments of the starch affects the diagram obtained. Katz and his co-workers from their work consider that air-dried starches contain water of crystallisation and that the staling of bread may be accompanied by crystallisation of the amorphous starch present.[41]

When starch is ball-milled or ground to destroy the granule structure the X-ray diagram is of the amorphous type.[102] L. H. Lampitt, C. H. F. Fuller and N. Goldenberg[103] have recently examined the changes in the X-ray spectrum of wheat starch which are produced by ball-milling the starch. Prior to grinding the interplanar spacing, ' *d* ', was found to be similar to the values found by Katz[105] and Naray-Szabó.[104] Every preparation examined by these workers exhibited a ' *d* ' value which is of the same order as that experimentally determined for the thickness of the molecules of three different trimethyl-β-methyl glucosides by Cox, Goodwin and Wagstaff.[113]

According to Meyer *et al.*[106] starch subjected to a pressure of 20,000 atmospheres gives an amorphous X-ray pattern presumably because the disruption of the inner arrangement of the crystallites in the granule structure.

As already mentioned the presence of a certain amount of water, probably of crystallisation, is necessary in order to get an X-ray diagram which is not amorphous, and from this it may be concluded that water is an essential factor in the structural formation of the starch granule.[107, 108] Katz and Weidinger[109] have shown that starch heated to 180-200° C. no longer gives the crystalline pattern, and it should be remembered that at this temperature not only is the loosely-held water present driven off but also that water which is not removed by maintaining starch at a temperature of 110° C. for a long time. The evolution of this moisture from well-dried starch is described in the chapter on Dextrin.

Lampitt *et al.* by extracting milled starch have obtained several fractions, differing in water solubility and described on p. 111,

The H.W.S. and the C.W.S. fractions gave different but definite X-ray patterns and if precipitated from their solutions by alcohol the resulting X-ray pattern differed from that given by the solids obtained by heat-drying. They thus conclude that the structure of the starch is greatly dependent on the way in which the starch is dried, and in the case of both the H.W.S. and C.W.S. fractions the X-ray pattern appears to be independent of the molecular weight of the fraction.

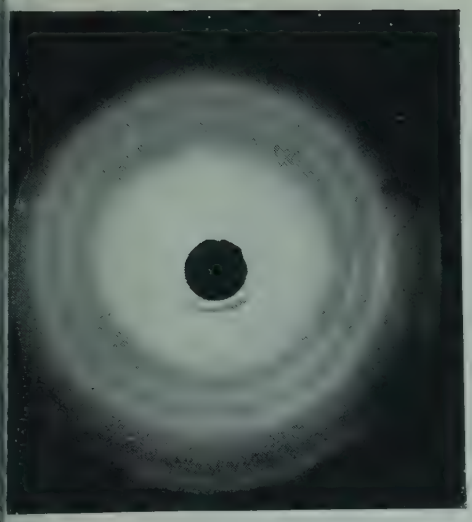
The X-ray pattern obtained with starch milled from 0-120 hours appears to be due to the H.W. insoluble fraction but beyond this time the contamination from the mill plays an increasingly important part until, after 1,200 hours' milling, any pattern obtained is due entirely to the silicates or other inorganic material from the mill. Between 300 and 470 hours' milling a change in the structure of the H.W.S. fraction takes place, as shown by the definite change in the X-ray pattern.

Figs. 4 : 2 A and B show the change brought about by grinding whilst C and D show that part of the X-ray pattern of the H.W. insoluble fraction is due to the presence of inorganic contamination by the mill materials. E and F show the differences between X-ray patterns of heat-dried and alcohol precipitated H.W.S. fractions, and if F and G be compared it will be seen that the X-ray pattern of the H.W.S. fraction, 470 hours' milling, differs from those of the other H.W.S. fractions.

The starch separating from solutions of H.W.S. and C.W.S. fractions had a much more definite pattern than that remaining in solution, thus confirming the observations of previous workers[108–112] and supporting their conclusions that starch pastes increase their crystalline character on ageing. Other work on starch has been reported by Samec,[30] and the X-ray technique has been used to study Schardinger's α-dextrin by O. Kratky and B. Schneidmesser.[31]

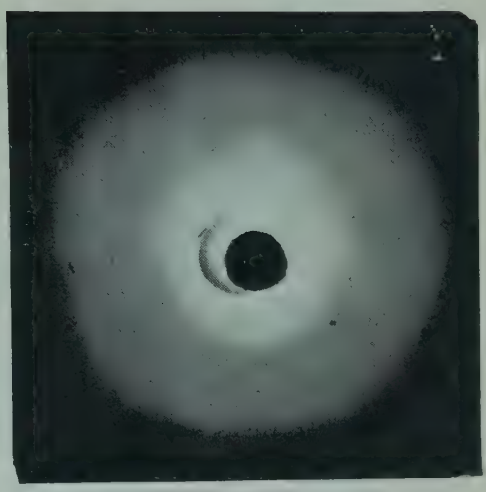
As we have seen X-ray and optical studies suggest that native, granular starches and retrograded starches with 'A' or 'B' X-ray patterns have essentially linearly extended chain configurations. Hanes,[8] and also Freudenberg and co-workers[9] suggest a helical configuration which explains the action of α-amylase and the occurrence of the achroic point in amylolysis when dextrins with 6 glucose units per molecule are produced. Also space models of starch permit helical models.

Freudenberg,[9] using the latter shaped spaced model, finds the central canal of the helix is large enough to admit iodine molecules and suggests that the blue iodine colour is due to the iodine being in this central canal which has a hydrocarbon lining, the OH



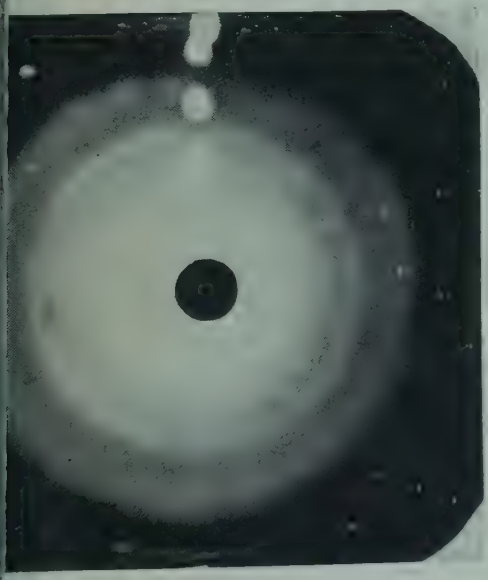
A

Unground wheat starch.



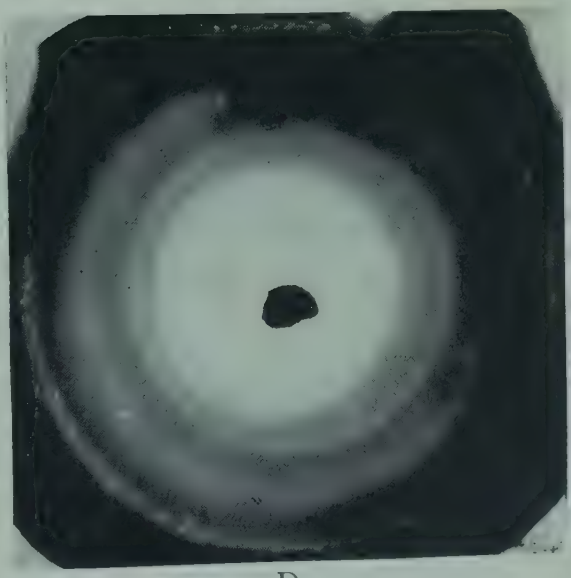
B

Starch ground for 120 hrs.



C

V. insol. 300 fraction (heat dried).



D

Ash from the H.W. insol. 300 fraction (C).

(Reproduced by courtesy of 'J. Soc. Chem. Industry'.

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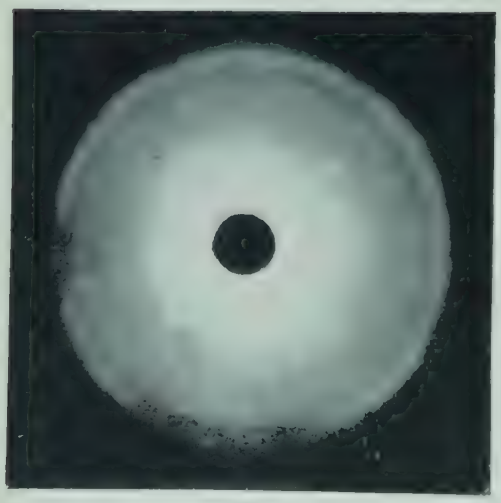
FIG. 4.2.



E

H.W. sol. 120 fraction (heat dried).

F

H.W. sol. 300 fraction (alcohol-precipitated).

G

H. W. sol. 470 fraction (alcohol-precipitated).

(Reproduced by courtesy of [illegible])

FIG. 4.2 (*continued*).

groups in the starch model being oriented outwards. Analogously iodine solutions in polar solvents are brown but in non-polar, i.e. hydrocarbon solvents, the colour is blue to violet. In the same way the iodine addition products with the cycloamyloses can be explained by a mechanism similar to that proposed by these workers for starch.

As there is incontrovertible evidence for the existence of extended chains in starch Meyer[16] objects to the above theory but Bear[12] has pointed out that both forms may co-exist. Meyer also points out that the helical model does not explain the colour of other iodine complexes which are known (see Chapter 10). Complex compounds such as various resins, polyvinyl acetate, polyvinyl alcohol, etc., have been noted by the writer to give iodine reactions varying from blue and violet to reds and browns, depending largely on the chain length or degree of condensation of the resin. It does not appear probable, therefore, that any one model is likely to explain the iodine coloration of widely dissimilar products (see Chapter 10) so that this is not a serious shortcoming of any model proposed for a particular iodine complex.

Rundle and Baldwin[17] find that starch-iodine solutions exhibit dichroism of flow, light with its electric vector parallel with the flow lines being more strongly absorbed than light with its electric vector normal to the flow lines. A velocity gradient, such as that produced by a rotating concentric-cylinder apparatus, would be expected to orient the long, starch-helices, having the associated iodine molecules located in the centre of the helices with their long axes coincident with that of the helices, preferentially along the flow lines. Such a state of affairs would bring about the result found by Rundle and Baldwin. Any other mechanism of adsorption of iodine molecules on to starch molecules which oriented the long axes of the former with the long axis of the latter would give a similar result. Thus dichroism itself is insufficient to distinguish between the helical configuration with the iodine molecules aligned along the central canal in the direction of their longer axes or an extended starch chain having adsorbed on to it iodine molecules with their long axes parallel to the starch chain.

Nevertheless, any acceptable structure suggested for starch must also explain the dichroism observed and this has not been true of some of the previously proposed structures for starch-iodide.

Unbranched, more nearly linear, starch chains should orient more readily during flow than branched chains and Rundle and Baldwin therefore use this criterion to examine the proposed

structures of various starches and starch fractions. Their results may be summarised as follows :

1. The butanol precipitated fractions from maize and potato starches show noticeable dichroism even in dilute solution indicating a linear unbranched structure.

2. The unprecipitated portion from butanol fractionation shows a doubtful dichroism and if the last traces of the blue-staining component are removed by Tanret and Pacsu's method, i.e. by adsorption on cellulose, no dichroism is observed, indicating a branched chain structure.

3. For ordinary blue staining starches dichroism is easily observed.

4. Glycogen-iodine complex shows no dichroism.

5. Waxy maize, glutinous rice or starches giving a red iodine reaction, in general, show little, if any, dichroism. These starches have recently aroused interest in that they appear to consist almost entirely of amylopectin or the branched-chain component.

The above work therefore provides entirely independent physical support for the proposed unbranched and branched structures for amylose and amylopectin, respectively.

Rundle and French have followed these findings with work aimed at distinguishing between the ' helix ' and the ' extended chain ' hypotheses. Frey-Wyssling[35] has pointed out that the optical properties of the helical model will be quite different from that of the extended chain model, such as is found in ' A ' or ' B ' modifications of starch. Schoch's production of rosettes of crystalline amylose by butanol fractionation[20] leading to the preparation, by Kerr and Severson,[19] of single amylose crystals by a modification of the method, permitted further work on the structure of starch.

Both the rosettes and the single platelets of amylose stain with iodine without apparent loss of crystallinity and therefore, presumably, without appreciable change in the configuration of the starch chains. If the starch-iodide involves helical starch chains the new crystalline products will have the required helical configuration. These workers find that both materials are very dichroic and consider the dichroism and birefringence cannot be interpreted in terms of starch-iodine complex consisting of extended starch chains. The optical properties, however, can be interpreted in terms of helical starch chains, with iodine molecules held in the central canals with their long axes aligned in the same direction as the canals, which are normal to the platelet face of the crystals.

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Chapter 5

THE SWELLING AND GELATINISATION OF STARCH

The Swelling of Starch.—Starch swells when treated with cold water, and the swelling is reversible. If the water be heated, the granules swell to many times their original volume and gelatinise. Thus on treatment with water starch exhibits two distinct phenomena :

(*a*) Real swelling in cold water, in which the absorption or loss of hygroscopic water is attended by swelling or shrinkage, and (*b*) Gelatinisation in hot water above certain temperatures, whereby the starch cannot be recovered from the suspension in its original form.

Ordinary air-dried starch contains a varying and considerable amount of moisture, depending on the origin of the starch and the humidity of the air. Wheat starch, on the average, contains about 13 per cent. moisture, whereas potato starch contains about 20 per cent. If starch is intensely dried, small fissures are sometimes observed in the granules, and the author has noted that these are very pronounced if the starch is dried in a vacuum. Samec has noted that such intensely dried starches are more susceptible to the influence of hydrolytic agents than ordinary unprocessed starch, and this may be ascribed to the easier access to the granule interior that these small fissures allow.

The reason why starch is insoluble in cold water but disperses in hot water appears to be connected with its crystalline structure. The crystals are arranged axially from the hylem but, although they are not touching, free access of water is probably prevented owing to the formation of oxygen bridges between the nearly parallel crystals. These bridges may be too strong to be broken by cold water, but raising the temperature may bring about the breaking of a few, allowing the starch to swell and thus throwing a heavier strain on the adjacent oxygen bridges which are then easily broken. Ling and Mehta have postulated the presence of a thin membrane of hemicellulose around each granule which may tend to restrict the swelling and the enzyme action. Gortner and Hamalainen,[87] from enzymatic studies on raw starch, also consider a membrane, but of protein, may surround the granules.

The hygroscopic water is very difficult to expel. Bloch[1]

states that a temperature of 155-160° C. is required to remove the last traces of water, and gives a method of estimating water based on the fact that a given weight of starch occupies a definite volume at maximum hydration.[2] If starch be left in a sufficiently damp atmosphere, it may take up as much as 36 per cent. water. When the vapour pressure of a substance which swells in water is measured and plotted against the amount of water present, the curve obtained is of the *f* type, and thus a ready means of studying the swelling of starch is available. With starch the curves so obtained on hydration and on dehydration do not lie one on the other, but show a lag behind the true amount of water present, i.e. the curves show hysteresis. The same phenomenon is shown by starch films, and can be examined by the technique of Farrow and co-workers.[35, 36]

F. Ullick[3] noted a rise of 3° C. when air-dried starch was mixed with its own weight of water, and a rise of 13·8° C. for a sample previously dried at 120° C. C. A. Winkler and W. F. Geddes[4] have also studied the heat of hydration or water-absorption of wheat starch and found that samples prepared from different wheats gave essentially the same heats of hydration. They separated potato starch into two portions, one containing all the large granules, the other all the small, and found that there was no significant difference between the heats of hydration of the two samples. They consider that the water actually permeates the granules of the starch. The heats of hydration of rice, wheat and potato starches were measured by these methods and found to vary widely at the same water-content and to increase in the order given. The critical moisture-holding content of tapioca starch has been shown by S. B. Etorma[48] to be 19 per cent.

The amount of heat liberated by wetting dried starch with water varies with the amount of water used, and if the heat evolved is plotted against the amount of water added a parabolic curve is obtained. From measurements carried out to ascertain the entropy changes in such a system, it may be shown that the first water added to starch is taken up in a regular manner as a hydrate, i.e. in the same manner as water of crystallisation, and this has led J. R. Katz and his co-workers[5] to suggest that this water is bound as in a zeolite, a suggestion which they also support with evidence obtained by their work on the X-ray spectroscopy of starch. G. Centola,[49] from his studies on the hydration of starch, considers that the molecules must have a zig-zag structure.

Sair and Fetzer[124] have examined the sorption isotherms for 7 commercial varieties of starch. They fell into 3 groups, viz. tubers (white potato) > pith or root starches (sago, arrowroot

and sweet potato) > cereal starches (wheat, maize and rice). They consider this can be taken as a measure of their degree of association, on the assumption that free hydroxyls must be available for the attachment of water. Up to 25-30 per cent. relative humidity all the starches take up the same quantity of water which may be related to the quantity theoretically required to form a monohydrate. Treatment with steam at 5 lb. per sq. in. reduces the difference in sorptive capacity and alters the X-ray patterns in the direction of the cereal starch pattern.

The swelling of starch granules in water at various temperatures below the gelatinisation point has been studied by W. Gallay and I. E. Puddington.[122] The dilatancy is particularly marked just above the freezing point and decreases with rise in temperature and the sedimentation volume increases correspondingly. The degree of compactness of the settled starch decreases with increase in temperature. At 10° below the gelatinisation point flocculation becomes apparent. Thus below the gelatinisation point there appears to be a continuous increase in hydration of the starch with temperature, this hydration being essentially reversible. If the starch is intensively dried before making the aqueous suspension it shows little or no dilatancy, a lower gelatinisation point and a higher sedimentation volume. The high heat of wetting of intensively dried starch, mentioned above, is suggested by these workers as the reason for the low dilatancy of this type of starch as it brings about incipient gelatinisation at the surface of the granules leading to sticking and aggregation thus increasing the sedimentation volume and destroying dilatancy.

The Gelatinisation of Starch.—On heating with water above certain temperatures, the various starches form viscous pastes. The ease of formation of these pastes often varies with the previous treatment of the starch, i.e. whether during manufacture it has been subjected to the action of acid or alkali in excessive amounts, or for excessive periods of time. The viscosity of such pastes is generally lower than normal, and allows of a more thorough agitation and dissemination of heat when the pastes are being made. The fact is of interest where the pastes are intended as adhesives, or for sizing yarn, or dressing textiles. A further factor in determining the viscosity of a starch paste is the drying-treatment accorded to the starch during manufacture. One which has been dried at a very low temperature generally shows a greater viscosity when made into a paste than one which has been quickly dried at an elevated temperature.[6]

When granules of potato starch are suspended in water and the temperature of the suspension slowly raised, they first swell

slowly, then, at a certain point, a small hole appears at the hilem. This hole does not make its appearance simultaneously in all the granules. The hole increases in diameter as the heating is continued, and the granule swells up to many times its original volume, and loses its birefringence. By the use of swelling agents, e.g. calcium nitrate or sodium hydroxide solutions, the rate of swelling of starch can be controlled at a speed which it is convenient to follow at leisure. The bubble grows until the granule has been weakened and starts to collapse and the bubble rapidly disappears[121] indicating that during the rapid swelling process the interior of the granule is a region of very low pressure, so much so that a void is formed and expands with the pressure of the water within the system. The swelling forces must be largely in a tangential direction to the granule surface since a radial expansion would not reduce the interior pressure.

When fully gelatinised the granule appears as a gelatinous sac or vesicle filled with an aqueous solution of amylose, which can be stained with iodine solution. The Brownian movement can be observed in the interior of the sac, and the solution gradually diffuses through the gelatinous wall. This is demonstrated by adding a concentrated tannin solution to a fresh preparation, when a precipitate is formed inside the sac, but a preparation several days old does not show this. When the heating is still further continued the sac bursts. The latter effect does not happen with all starches, but can readily be observed in potato starch, which gives a solution containing amylose and particles of the sac in suspension. Granules of wheat starch do not appear to burst or lose their identity ; the paste made from this starch remains full of large gelatinous vesicles, and thus explains to some extent why wheat-starch pastes do not penetrate textiles but tend to lie on the surface. It also explains the variations in viscosity obtained from starch pastes, especially from potato starch, on stirring ; the structure of the granules is being broken down, and so they take up less space and the viscosity falls. Bear and Samsa[121] interpret the swelling and gelatinising phenomena shown by the granules as an actual contraction and thickening of radially oriented starch molecules rather than ordinary osmotic or hydration effects alone. To observe the swelling of starch when heated with water under the microscope, a suspension of one part of starch in two hundred parts of water is very convenient. The examination may be carried out by heating a suspension and examining withdrawn drops under the microscope, or the suspension on the microscope slide may be heated electrically,[7] or by means of a current of hot water[8] in contact with the underside of the slide. Different

workers give varying temperatures at which the various starches gelatinise; this is not only due to the different conditions of experiment, but also because some workers take the gelatinising point as that at which anisotropy disappears from all the granules, whilst others take it to be when the majority of the granules show loss of anisotropy. L. Heintz keeps suspensions of starch at different temperatures and then filters. When the filtrate shows a blue iodine colour he considers the starch has commenced to gelatinise. Thus he finds rice starch starts to gelatinise at 55° C. and that the process is complete at 75° C. Furthermore, starches from different varieties of the same species of plant often show variations in their gelatinisation temperature—thus Alsberg and Rask[9] found this to be the case with wheat and maize starches, and their observations on the latter starch have been confirmed by Dox and Roark,[7] who examined thirteen varieties and obtained gelatinisation temperatures ranging from 64·1° to 71·1° C. S. G. Willimott[50] has studied the gelatinisation temperatures of maize, potato and colocasia starches. Alsberg also found the viscosity of the mucilages prepared from the different varieties to vary considerably. It should be emphasised that every granule of starch has its own gelatinisation temperature, which may be called its 'specific gel temperature', at which it is fully swollen and no longer shows anisotropy. In the same starch suspension the larger granules appear to swell at a lower temperature than the smaller granules, and it is interesting to note that, other factors being equal, a delivery of starch with a smaller average particle size than normal generally requires a somewhat higher temperature for conversion to dextrin and gives a more opaque paste unless boiled for a considerable time, whilst a delivery having a great majority of larger sized granules than usual is more readily converted.

The Use of Swelling Agents to Study Gelatinisation.—Instead of heating a starch solution to observe the swelling, use may be made of the effect of certain salts and alkalis which lower the specific gel point so much that at ordinary temperatures the effect can readily be obtained and observed. The following compounds,[10] in the percentage concentrations stated, can be used for microscopical observations at normal temperature; sodium hydroxide 0·53, potassium hydroxide 0·75, potassium thiocyanate 12-15, potassium iodide 26-28, ammonium nitrate 30-35, silver nitrate 29, chloral hydrate 55. Other salts that may be used are calcium nitrate or chloride, sodium or potassium bromide, or ammonium thiocyanate.

Granules of potato and of canna starch treated with concen-

trated ferric chloride solution swell at once and reach a maximum degree of swelling in 10 minutes. With nickel chloride solution the swelling begins after a lag period of several minutes and reaches a maximum after 1 hour. Cobalt chloride has an initial lag period of 30 minutes but even after 24 hours the swelling is still incomplete and individual starch granules can still be detected.[116]

Iodine penetrates the starch granules very rapidly without swelling and sodium thiosulphate will decolorise these stained granules just as rapidly. This indicates a high degree of porosity to both solutions.

Use has already been made commercially of the properties of these substances to produce special starches for various purposes, and further reference will be made to them in the appropriate sections. The addition of soap to the water used for swelling starch raises the gel point progressively, and if sufficient is added it inhibits the swelling completely. Penick and Ford[38] have patented the use of soaps, sulphonated tallow, vegetable oils, and hydrogenated phenols, as inhibitors of swelling, to obtain a modified starch. The starch is heated in water containing the inhibitor until the anisotropy of the granules disappears. The swelling of starch and of gelatine also has been studied by J. R. Katz,[11–13] using swelling agents of the organic type, i.e. thiourea, resorcinol, benzene, sulphonic acid or chloral hydrate, etc. He finds that compounds containing two hydrophilic groups show a somewhat weaker swelling action than those containing only one, and ascribes this to the weaker adsorption of the former. In the presence of a single hydrophilic group the swelling action on potato starch is in the order phenol > aniline > benzoate > benzene sulphonic acid. The length of the molecule also appears to play some part in the swelling action where two compounds of the same empiric formula, the same molecular weight and containing the same hydrophilic group, are concerned. Thus para-diphenyl sulphonate exerts a much greater swelling effect than naphthalene-sulphonic acid. With alkylamine sulphates and bromides a ' lyotropic ' series of the alkylamine cations is obtained, and the curves show that the effect is stronger the longer the alkyl group ; and the preventive effect of the sulphate is weakened, the greater the number and lengths of the alkyl groups in the amine. The effect of the amine-group is additive to that of the sulphate. The greatest effect ever observed by Katz and co-workers[39] is given by triheptylamine bromide.

J. R. Katz and J. Seiberlich[70] have examined the influence of mixtures of lyotropic agents on the pasting temperature and

showed that, with one or two exceptions, the curve for a mixture came between those for the components. The clear, viscous solution obtained by the action of formamide on starch has been studied by several workers.[72]

The endothermal effects during the gelatinisation of potato starch suspensions in sodium and potassium chloride solutions and in various acids have been studied by V. I. Nazarov and A. V. Nikolaev.[71]

Starch appears to follow the same rules as gelatine and the proteins as regards swelling brought about by the presence of salts. The influence of the anion on the swelling is marked and unmistakable, some anions merely producing a slight swelling whilst others act so powerfully as to cause the starch to swell and gelatinise in the cold. C. E. Mangels and C. H. Bailey[14] used the hydroxides, thiocyanates, iodides, and chlorides of potassium and sodium, and some of the alkaline-earth salts, and followed the swelling by determinations of the viscosity of the suspensions, using the Ostwald-pipette type of viscometer. In general, the swelling of the starch increases with the concentration of the salt present and a considerable variation in the power of the different reagents may be noted. The anions fall into a Hofmeister lyotropic series, being arranged in the order: Cl < Br < I < CNS < salicylate < OH. If the effect of the cations be examined, it is found that they exhibit some effect other than that shown by a lyotropic series. The presence of small amounts of electrolytes, even in such small amounts as are present in tap water,[15] affects the viscosity of starch pastes adversely; and E. Wiegel[16] has also shown the disproportionate effect on the viscosity of these pastes exercised by minute amounts of electrolytes.

J. R. Katz and co-workers[47] have recorded the relationship between the pasting temperatures of potato starch and the molar concentrations of electrolytes and various acids added to the water. They examined the chlorides of NH_4, Li, Na, K, Be, Mg, Ca, Sr, Ba, Al, and those of their sulphates which are soluble, and the thiocyanates. Beryllium chloride was without effect in the range examined (0·5-3·5 molar), but all the other chlorides depressed the pasting temperature. All the sulphates increased the pasting temperature, but the thiocyanates had a remarkable depressing effect.

Depression of the pasting temperature sets in at the following molar concentrations of acid: with nitric and hydrobromic about 0·5; hydrochloric, about 0·8; phosphoric, about 1·7; and sulphuric, about 4·5. All the organic acids cause depression;

the effect of citric, tartaric and acetic acids was slight, but a strong effect was noted with the chloroacetic acids which increased with the number of chlorine atoms.

As will be discussed later, on heating a starch suspension to the boiling-point, and maintaining it at that temperature for some time, a rise in viscosity to a maximum is observed, followed by a fall to a minimum, but ultimately rising to a final value on cooling it. R. A. Gortner[17] has studied the gelatinisation of starch, and considers that the continual boiling greatly reduces the hydration of the starch, thus leading to a fall in viscosity. He thinks that this explains why the viscosity of potato-starch pastes falls more rapidly than that of wheat starch. It must be remembered, however, that the gelatinised vesicle of potato starch is more fragile than wheat starch, and therefore much more readily broken down, leading to a decrease in viscosity. The above worker considers that gelatinisation in the cold, using swelling agents, differs from gelatinisation brought about by heating, but that the mechanism of the cold swelling is the same whichever agent he used. The gelatinisation involves at least three stages according to this worker. At low concentrations of the chemical no hydration takes place, but as the concentration is raised there is a rapid increase in the swelling of the granules, and finally rupture takes place followed by the peptisation of the particles thus produced with a concomitant increase in volume. As the concentration is still further increased, a point is reached where it causes a slow osmotic dehydration.

Mullen and Pacsu[123] have studied swelling in aqueous pyridine solutions. Gelatinisation, as measured by consistency, does not occur until the pyridine content exceeds 60 per cent. and the gel temperature, i.e. that at which maximum viscosity is shown, is lower in 10-50 per cent. pyridine solutions than in water alone. The viscosity at the respective gelatinising temperatures is greatest with a pyridine concentration of 50 per cent. and least with 30 per cent. A 30 per cent. pyridine solution always gives a smooth and transparent paste probably because disaggregation of the granules is greatest at this concentration. The heat of gelatinisation in water decreases in the order, potato, tapioca, wheat, maize and rice, i.e. in order of decreasing granule size and increasing gelatinisation temperature. In 30 per cent. pyridine similar results are obtained, although the temperature range for gelatinisation is smaller, but the maximum viscosity decreases in the same order. Wheat starch, which has a great range of particle sizes, behaves somewhat anomalously, as the present writer can confirm. These workers consider the heat of gelatin-

isation is more than sufficient to break one or two hydrogen bonds per glucose unit.

Methods of Following the Course of Gelatinisation of Starch: Optical Methods. (See also Chapter 19, Vol. II), by Kesler and Bechtel.

When a starch granule gelatinises four phenomena occur: swelling, loss of anisotropy, the power of injured granules to take up certain dyestuffs,[18] and solubilisation. Several workers have explained anisotropy by postulating that there are small crystals in starch which are subjected to internal strains, and that these strains are relieved when the granule gels, so that anisotropy disappears.

Swelling, loss of anisotropy, and staining of the granules with dyestuffs, such as Congo red, can be followed visually, but as about 300 granules have to be examined at each reading, and such readings are not continuous, points like these constitute a drawback to visual methods. Such methods have, however, shown that each granule has its own temperature of gelatinisation, and therefore it is not sufficient to obtain the gelatinisation point of a sample of starch in order to differentiate between different starches.

If the temperature of a starch suspension be increased slowly at a constant rate, samples may be withdrawn at successive increments of time, and the temperature need not be measured directly. If such heated samples be examined under a microscope fitted with crossed Nicol prisms, those grains which have lost their anisotropy can readily be seen except where they are too small to show this effect. Carrying such an experiment to its end-point, we should finish with all the granules gelatinised, and if the number of granules gelatinised in each sample is plotted against the time of heating of the sample, and hence against the temperature, since this increases at a constant rate, an S shaped curve is obtained, as shown in Fig. 5 : 1.

At the point A on this curve only the very largest granules have just started to gelatinise, whilst at the point B the largest of the granules of average size have begun to lose their anisotropy. From point B to C the curve shows that within a short period of time, or a narrow temperature range, the granules of the average size forming the bulk of the mass have gelatinised, and from C to D the smaller granules are affected. The temperature at C corresponds to the gelatinisation point, as recorded by those workers who take the point at which the anisotropy disappears from the majority of the granules.

This curve gives some information about the previous history

of the starch, and it will be found that although tapioca starches from different factories give the same form of curve, the gradients are different. A starch that is separated by sedimentation and of first quality will show a steep gradient, whereas a starch separated by centrifugal methods has a less steep gradient, and is spread out more. Fig. 5 : 1 shows the *type* of curve for starches of this kind. This may be expected, as a centrifuged starch contains all the granules of all sizes, each having its own gelatinisation point, whereas many of the granules of a first-class sedimented

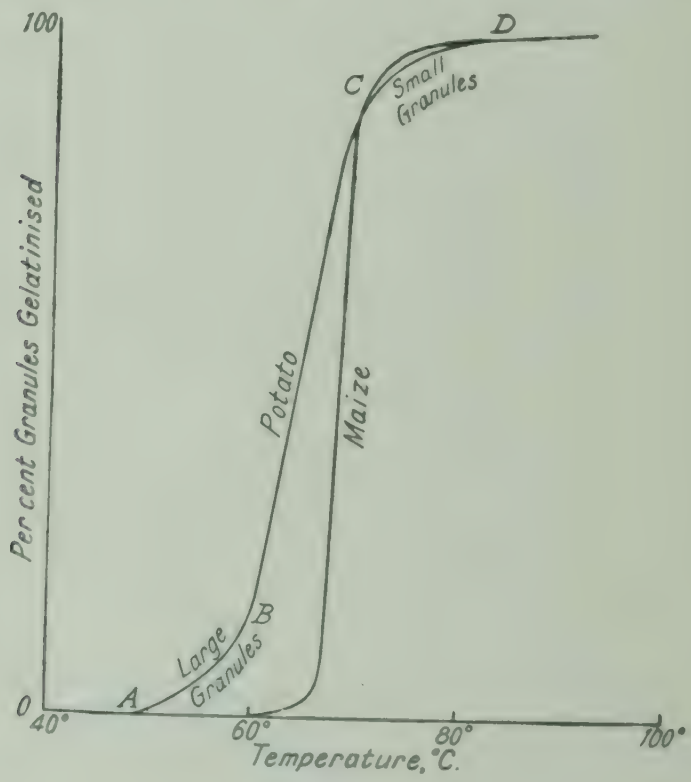

Fig. 5 : 1.

starch are large and the size is more uniform, so that they all gelatinise within a narrow range of temperature.

As the size of the granules appears to influence the speed of dextrin formation in the roasting process, this curve also gives information about the ease with which roasting can be brought about ; the steeper the curve the less complicated the process.

As injured starch granules can be stained with Congo red, this property can be utilised to follow the gelatinisation, instead of using the loss of anisotropy as the criterion, and the same type of curve is obtained as before.

Another method of following the gelatinisation of starch is to suspend 5 gm. of starch in 100 ml. water, heat at a definite rate to a definite temperature, cool, make up the volume of the suspension to 250 ml., and then allow the suspended matter to settle at 15° C. in a graduated cylinder. When settlement is complete, the volume of the settled material is read and plotted against the temperature. This is repeated with a number of samples and curves similar to those shown in Fig. 5 : 2 may be obtained. The curve marked A represents the type given by potato starch, and it will be seen that it rapidly reaches its maximum volume, and therefore at this point the viscosity of the paste is in the region of the maximum obtainable for this figure. The curve B, which shows the type given by wheat starch, is much more extended, and conforms with the fact that pastes of this starch take longer to reach their maximum viscosity.

Another method but recently introduced by Cook and Axtmayer[65] makes use of a photoelectric cell to measure the increase in the amount of light passing through a starch suspension as it is gradually heated.* A 60-watt tungsten lamp is used as the source of light, fed from a Delta transformer to smooth out line voltage fluctuations. The light is focused by a condenser lens on to a flask containing a suspension of the starch under examination. The flask is wide-necked, fitted with a thermometer and a stirrer, and is immersed in water contained in a litre pyrex beaker, which is heated by a three-stage hot plate. The beaker has around it black paper in which are cut two holes, diametrically opposed, of 3·75-5 cm. diameter, through which the light enters and leaves. On leaving, the beam is caught by a copper oxide or photronic type of photoelectric cell, connected directly to a microammeter reading from 0-50 microamperes.

To make a determination the water in the beaker is heated to about 40° C., 1-2 gm. air-dried starch suspended in 25 ml. water is poured into the flask, and the temperature adjusted so that it rises 0·5° C. per minute. Observations are taken from this point until the readings of the microammeter have become constant or boiling-point is reached.

The readings may be plotted to give smooth surves (see Fig. 5 : 3), since the current in the cell is a linear function of the light transmitted, and the authors call the temperature at which an increase in transmission of light is shown the 'transition temperature'. This point and the rate of gelatinisation, as shown by the curves, are reproducible and characteristic of particular samples of starch. They may be used to identify the starch if

* See p. 94.

the same process of manufacture is used for the sample and the standard, but treatment of a particular starch with acids or alkalis.

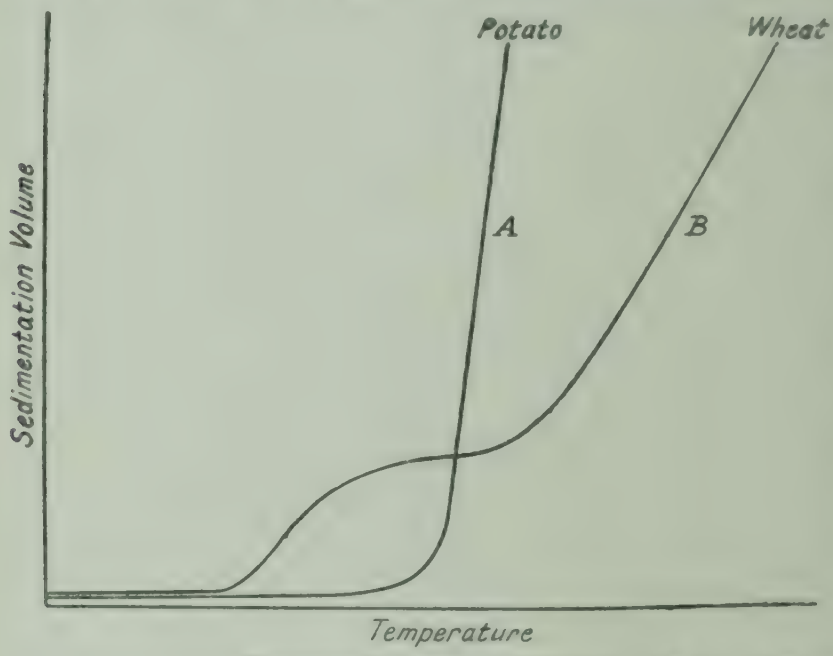

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Fig. 5:2.

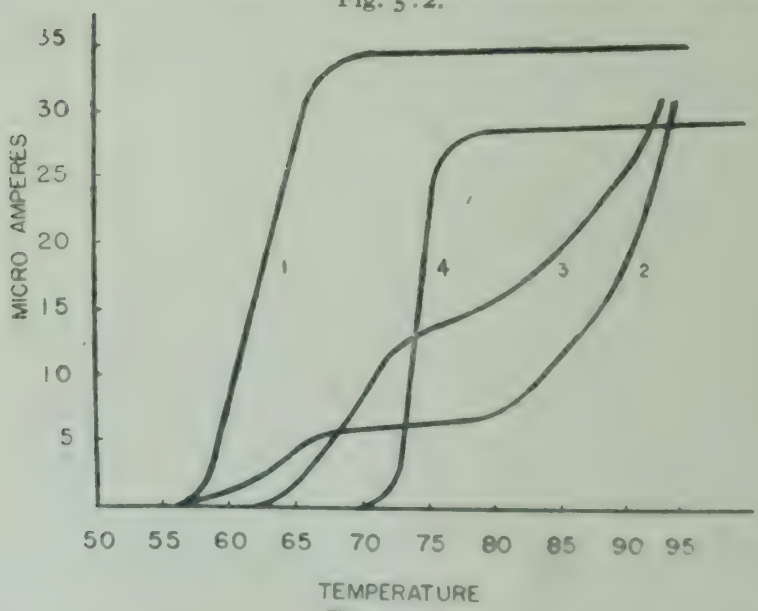

Fig. 5:3.

or drying over desiccating agents, or at 50-60° C., modifies the temperature of transition and the slope of the curve.

A constant rate of heating should be observed, as these workers

find that this factor affects the transition temperature but not the slope of the curve; thus by changing the rate of heating from 6·3 to 0·8 minutes per degree rise in temperature, the transition temperature of a cassava starch was raised 3° C. The swelling of starch may therefore lag behind the temperature when this is raised too quickly; the author has also found this to be the case, more especially with maize and rice starches.

Yantia starch was separated into two fractions having an average particle size of 4 and 12 microns respectively, but no difference was observed between the two curves obtained from these fractions. The above workers consider, however, that small differences might be shown by the curves obtained from the large and small granules of wheat starch. The fact that taro starch, with an average particle size of 2 microns, gives exactly the same type of curve as edible canna starch is held by these workers to indicate that the granule size has no influence on the rate of gelatinisation. The large and small granules may have different temperatures of transition, but apparently they gelatinise and rupture at the same rate.

Another point of interest in this work is that tropical starches were found to differ characteristically from northern starches. Northern or temperate-zone starches showed a transition temperature between 55° and 65° C., and gave gently rising curves showing a moderate increase in transmission, followed by a latent period or slowing down of the rate of increase of transmission between 65° and 85° C., and finally exhibiting an increased rate of transmission up to 90° to 95° C. On the other hand, tropical starches show a transition temperature between 60° and 78° C., with curves rising fairly steeply until the transmission becomes constant, which occurs most often at a temperature below 85° C. Fig. 5 : 3 shows in a diagrammatic way the types of curves obtained with arracacha (1), wheat (2), potato (3) and taro (4) starches when examined by this method.

Cook and Axtmayer confirm the observations previously made by Sjostrom,[32] Taylor, and Schoch,[31] that granules of tropical starches rupture completely below 85° C., but that with white potato, wheat, barley, maize, and rice many swollen but unruptured granules may be observed in their pastes even at temperatures between 90° and 95° C., a peculiarity which they attribute to the fragility of the outer sac of tropical starches. They found that a plant can acclimatise itself in such a way that the starch giving one type of curve tended to give the other type when grown in a different zone, so that a variety of potato grown in the north yielded starch which gave a curve of the temperate-zone type, but when the same variety was cultivated in a tropical zone

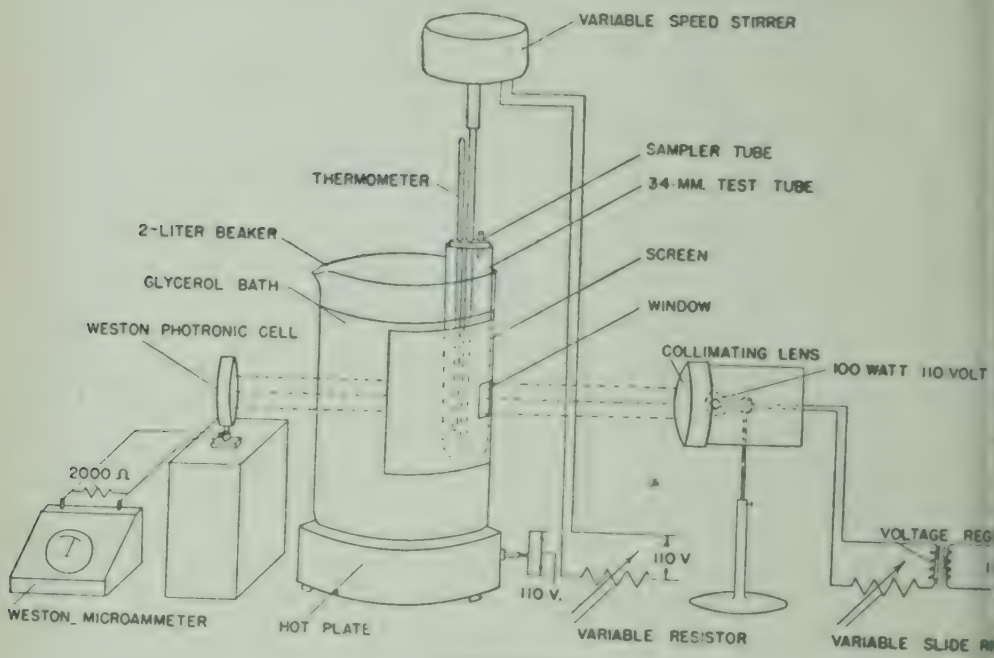

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Fig. 5:4.—Photopasting apparatus.

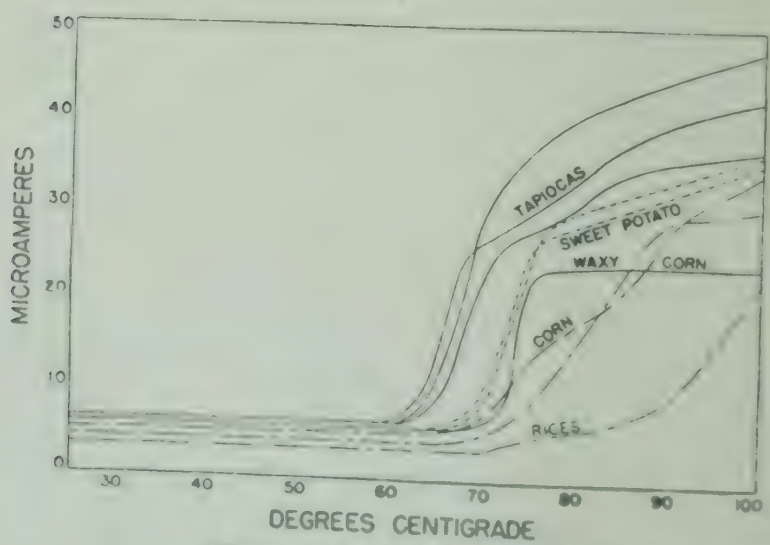

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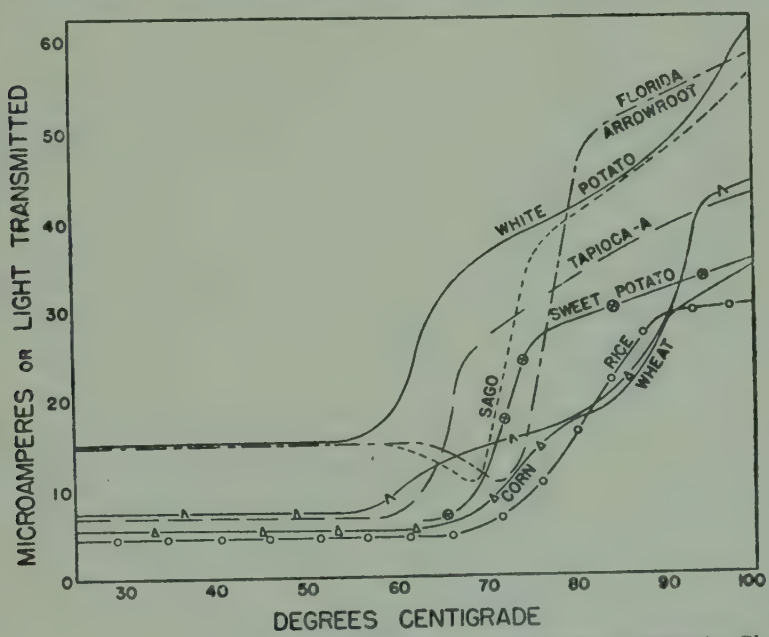
Fig. 5:5.—Variations in raw starches.

the starch obtained from it gave a curve tending towards the tropical zone type.

W. L. Morgan has developed Cook and Axtmayer's method, and Fig. 5 : 4 shows the apparatus used. The test requires only 0·33 gm. of sample, the starch being suspended in 65 c.c. of water contained in the large test tube, the stirrer and the glycerol-heater started and the rate of heating adjusted to give a rise of 2·5° C./min., so that a test is completed in about 30 minutes.

The various raw starches give characteristically different



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Pasting begins at: white potato, 55°; wheat, 56°; tapioca A, 61°; rice, 63-70°; corn, 64°; sweet potato, 64-67°; sago, 69.5-70°; Florida arrowroot 72° C.

Fig. 5:6.—Pasting of raw starches.

transparency/temperature curves (see Fig. 5 : 5), and Morgan considers that easy and positive identification of raw starches is possible owing to the characteristic shape and location of the curves. The larger grained starches at the low dilution of 0·5 per cent. are initially less opaque than the smaller grained cereal starches (Fig. 5 : 6). Just before the onset of pasting the breaking apart of small grains of sago and arrowroot into the individual granules causes a small increase in opacity and this behaviour is characteristic of these and other sticky types of starch. Tuber starches show steeper curves than grain starches, owing to the

shorter gelatinising range, and they show the greater transparency characteristics of pastes from this type of starch.

Wheat starch has two types of granule, the large readily pasted type and the small more resistant type, and this is brought out by the pasting curve for wheat starch. According to Fig. 5 : 5 the grain size distribution of raw rice starches has a definite effect on the pasting curves, which is what might be expected.

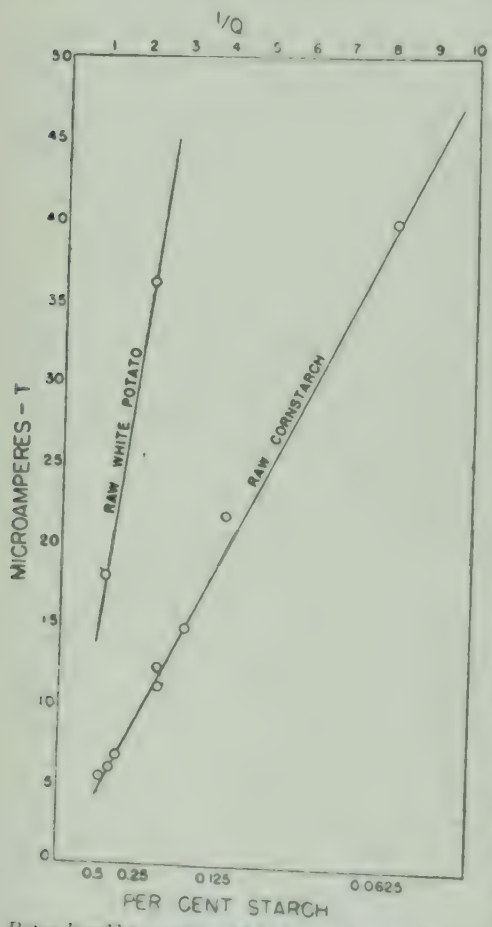

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Fig. 5 : 7.—Opacity of unpasted starches.

Duplicate tests by this method give curves in very close agreement. 'Waxy' corn starch gelatinises completely over a very narrow temperature range below 80° C., showing that it is very desirable for certain types of work, and its curve shows that in paste characteristics it is very similar to sweet potato and tapioca starches. The pasting curves of the several grades of raw tapioca starches show variations from each other although they are of the same type.

Fig. 5 : 7 shows the relationship of transparency to the amount of unpasted starch in suspensions of different concentrations, and it will be seen that it is a hyperbolic or inverse ratio function. This also holds for the simpler pasted starches. Acid thin-boiling starches (Fig. 5 : 8) give the same form of curve as raw starch but the initial pasting temperature and general location of the curve have, however, shifted upwards. Lower initial pasting temperatures and greater final transparency is, however, given by dextrins, the shifts altering progressively with increase in the degree of conversion. It is interesting to note that the 90 thin-boiling starch, which has also been dextrinised, gives a curve indicative of both treatments. Tapioca dextrins and oxidised starches (Figs. 5 : 9, 5 : 10, 5 : 11) likewise give pro-

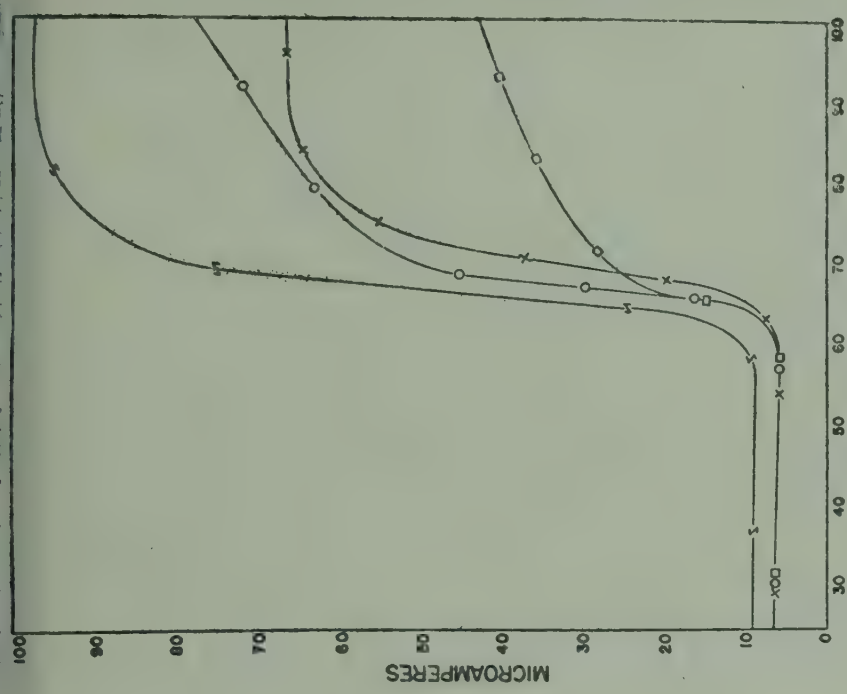

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Fig. 5:9.—Pasting of tapioca dextrins.

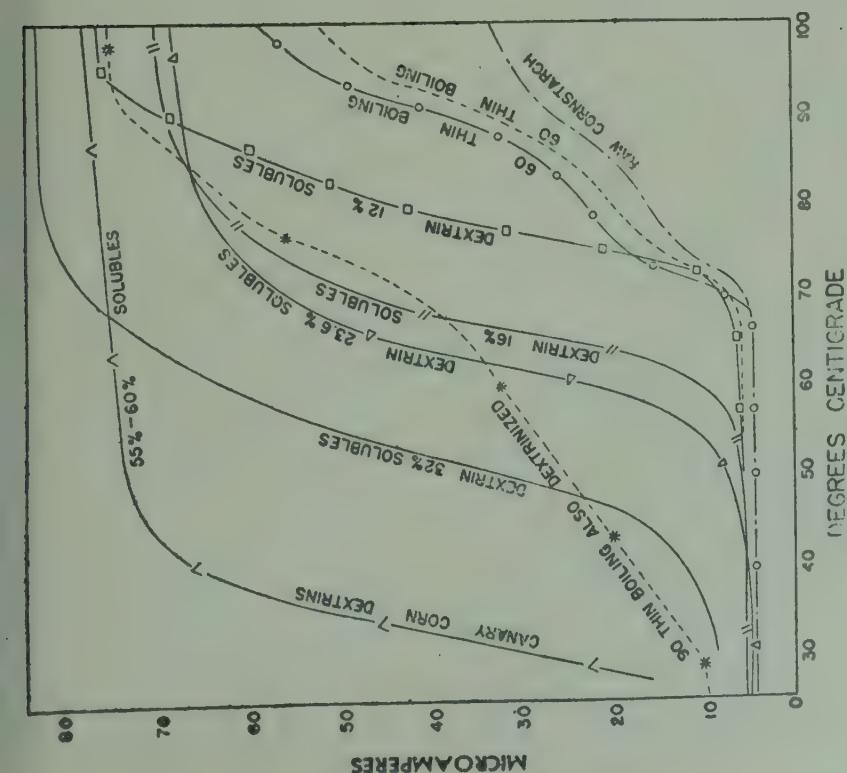

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Fig. 5:8.—Corn dextrins and thin boiling starches.

gressive shifts in the location of the curves as the modification is increased. Fig. 5 : 12 illustrates the use of this method to determine the approximate composition of a mixture of starches. It is assumed that the curve for the sample is the additive resultant of the components, and as the reciprocals of the light transmission are proportioned to the amounts of starch present, the composition can be calculated from these quantities. The corn and wheat curves cross at 80° C., i.e. have the same transmission at this point, so that they may be regarded as a single component so as to calculate the amount of tapioca starch present. The curve of the sample will, therefore, deviate from this point in a manner depending on the amount of tapioca starch present. The difference in the reciprocals of transmission at 80° C. allows one to estimate the percentage of tapioca starch, this being related to the extreme difference in reciprocals of the corn-wheat point at 80° C.

Thus a figure of 43 per cent. tapioca is indicated by the transmission values at 80° C., or if the point at 89·5° C. is taken, where wheat or corn curves again show the same transmission, a value of 45 per cent. is obtained. In the same manner at 66° C. the curves for tapioca and the unknown sample cross, and the maize can be calculated and a figure of 40 per cent. is obtained. The curve of a mixture of 40 per cent. tapioca, 40 per cent. corn and 20 per cent. wheat starch is close enough to show the validity of the above method for matching unknown samples.

The method is only applicable where swelling but not solution or break up of the granules is encountered, as in the case of dextrins. Fig 5 : 13 shows that true dextrin of 20 per cent. solubility is easily located, but a mixture of a higher converted dextrin with raw starch does not lend itself satisfactorily to the calculation of composition.

A photometric method of measuring changes in the opacity of starch gels has been used by C. A. Glabau and P. F. Goldman[40] in their studies on the staling of bread. A. J. Ophof[41] considers that the transparency of cassava-starch gels depends on the grain sizes as well as on the concentration, and that the temperature of swelling depends on the concentration.

A. Küntzel and K. Doehner[63] devised an apparatus which automatically records the temperature and the light absorbed, and consider that paste formation takes place in two phases. At the first temperature they consider that a 'melt' is formed of the starch crystallites together with deformation of the 'fibre' molecule. At the second temperature the hydration of the starch molecules, discharged from their lattice formation, sets in and swelling occurs. They also measured the heat liberated when

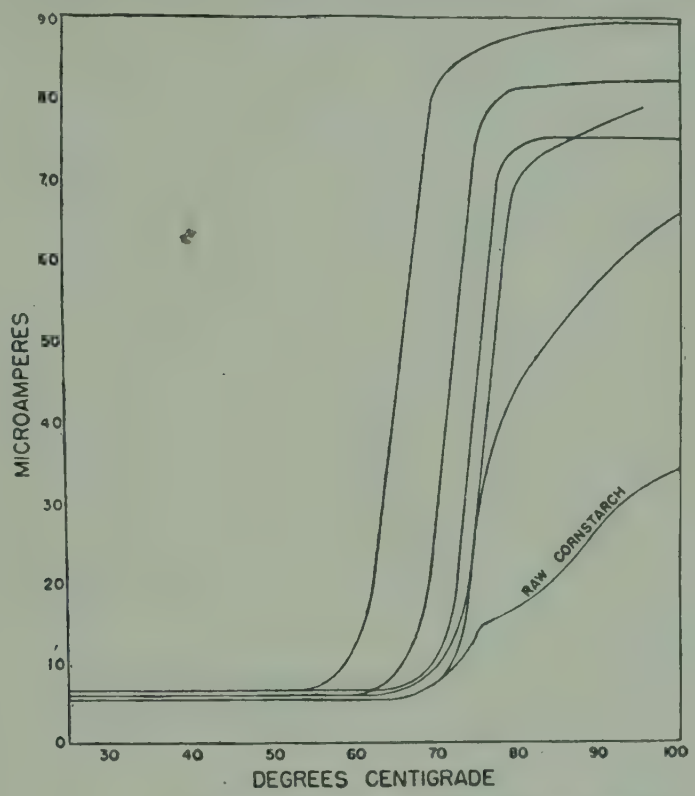

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Fig. 5: 10.—Pasting of oxidised corn starches.

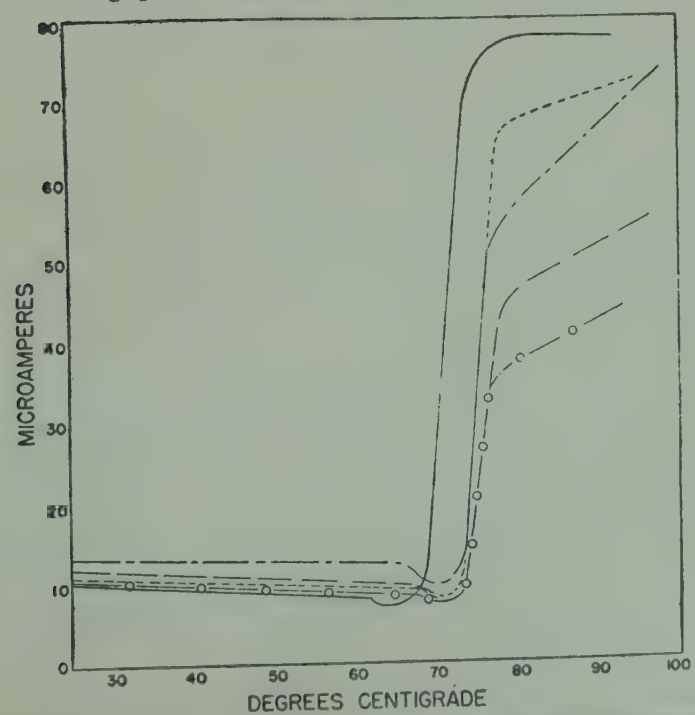

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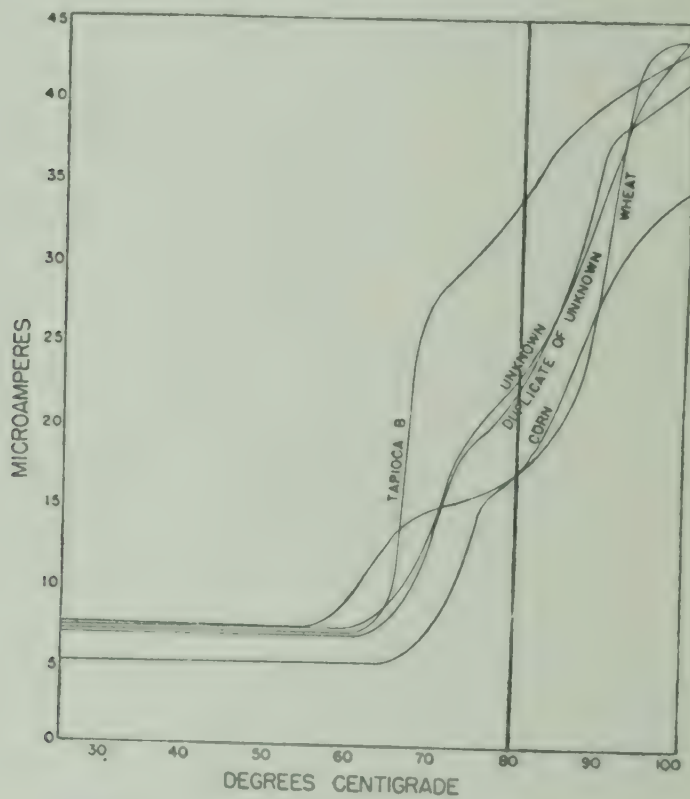
Fig. 5: 11.—Pasting of oxidised tapioca.

starch pastes were formed and for potato starch this was − 36·75 calories per gm. of dry starch. It includes the heat produced during the 'melting' and deformation stage (endothermic) amounting to − 39·35 cals., and that liberated during hydration (exothermic) amounting to + 2·6 cals. The heat formed during



At 80° C.	T	$1/T$	
Raw wheat } Raw corn }	17.0 ma.	0.0588	0 0588
Raw tapioca	33.0	0.0303	
Extreme difference		0.0285	
Unknown sample	21.5	0.0465	0.0465
Lower difference			0.0123

$$\% \text{ tapioca} = \frac{0.0123}{0.0285} = 43\%$$

Duplicate of unknown: 20 raw wheat, 40 raw corn, 40 raw tapioca

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Fig. 5:12.—Analysis of mixed starches.

the swelling of dried starch prior to paste formation was 17·47 cals. Katz and Seiberlich[62] consider that in the formation of a paste the starch, when containing a definite excess of moisture, is transformed into another modification which then absorbs water and swells, and X-ray evidence has been obtained by these workers to support this suggestion. W. W. Lepeschkin[64] also

supports the two-stage swelling theory, but suggests that hydrates are first formed and these then swell.

Methods Depending on Viscosity.—The characterisation of starches by viscometric methods is fully and critically dealt with by Drs. C. C. Kesler and W. G. Bechtel in Chapter 19, Vol. II, and is of especial interest to both the factory and the research chemist, so that, here, we need only develop the subject in so far as

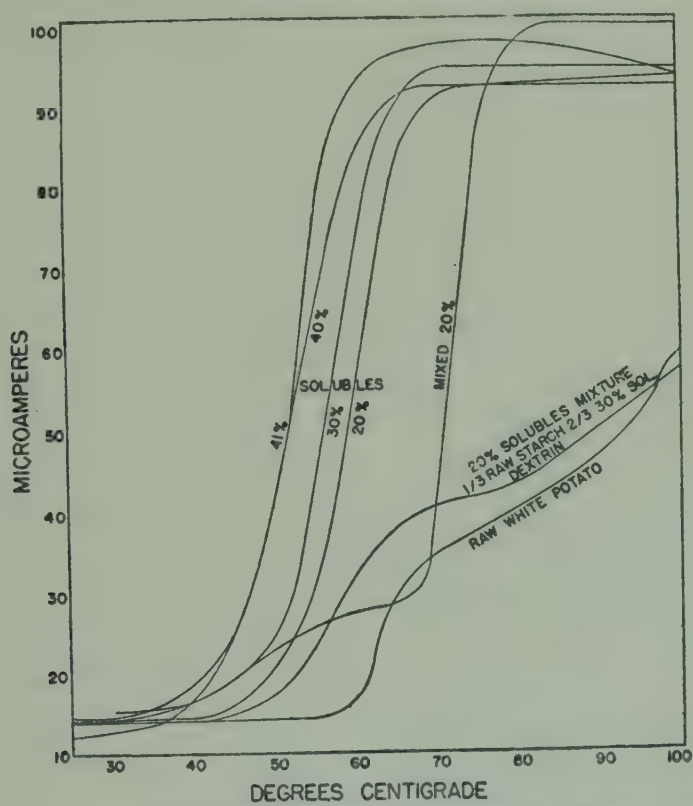

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Fig. 5:13.—White potato dextrins.

the behaviour of heated starch suspensions is connected with the sum of the behaviour of individual granules. When starch granules swell and gelatinise they occupy a much larger volume in the system than before this process took place, and hence the viscosity alters more or less with the swelling. Einstein[45] deduced a formula for the viscosity of a suspension of rigid spheres in any liquid in terms of the viscosity (z) of the pure liquid and the ratio (f) of the volume of the suspended matter to the total volume. This was

$$z_1 = z(1 + kf),$$

where k is a constant, which Einstein first took to be equal to 1, but afterwards made equal to 2·5. A similar formula was deduced by Hatschek[46] for cases in which the suspended material occupies less than 40 per cent. of the total volume. In this case $k = 4 \cdot 5$. This formula was tested by Harrison[19] for swollen starch and water, and found to hold fairly well up to 30 per cent. concentration. The viscosity depends only on the volume of dispersed phase and not on the degree of dispersion.

Harrison[19] has examined the connection between the volume occupied by the swollen granules and the viscosity of the paste or solutions so obtained, working on lines similar to those previously mentioned. He carefully heats a 1 per cent. suspension of starch to a few degrees above the point of gelatinisation of the sample, care being taken not to disturb or break down the structure of the suspension by shaking or vibration. After cooling to 16° C., 15 ml. of the liquid are transferred to a graduated centrifuge-tube, and after centrifuging the volume of the granules is read off. The viscosity of a separate portion, compared with water at 16° C., is also determined, and Harrison finds that the viscosity is equal to 4·75 times one-fifteenth the volume of the granules, as read off the centrifuge-tube, plus unity. Harrison found that maize, wheat and rice starches showed a slight increase in viscosity with the length of time of heating, sago starch showing a slight decrease and potato starch, after rapidly acquiring its maximum viscosity, a decrease which takes place in an irregular manner, according to the amount of stirring during the preparation. As the vesicles of the potato starch are comparatively fragile and easily broken, the determination should be repeated several times. This method serves very well for determining the comparative stiffening powers of different samples of the same starch, but he considers that it cannot be used for comparing different starches, owing to the different times of heating, the different temperatures required to obtain the pastes, and the effect of heating on the viscosity.

H. Geinitz[69] has studied the times of flow, through a 'Tsuda' capillary tube viscometer at 20° C. and with various applied pressures, of 0·2 per cent. solutions of various types of the potato starch and potato starch products. He finds that soluble starch solutions obey the Hagen-Poiseuille law, their viscosity not changing with ageing but that chemically treated starch deviates somewhat from the law whilst the greatest deviation is shown by untreated starch. The two latter types decrease in viscosity with age and finally reach a steady value.

G. V. Caesar[20] has studied the problem of the viscosity of starch pastes in a different way, and his results are of especial interest to

the practical man dealing with pastes containing 10-20 per cent. of starch. The determination with any degree of accuracy of the viscosities of pastes of these concentrations at normal temperature is limited, and Caesar's work is of interest in that it follows the various changes in the viscosity of starch paste throughout the whole heating up, boiling and cooling processes to which many starches are subjected commercially.

Briefly, he utilises the amount of work done in stirring a starch paste as the measure of viscosity of that paste. The starch suspension is heated carefully, and slowly, up to the boiling-point, and is stirred by a stirrer attached to an electric motor. The electrical input into this motor is a function of the work done by the stirrer, i.e. of the resistance of the paste to stirring. The electrical current is measured by a wattmeter, and in his paper Caesar plots the current consumed against the time, and therefore the temperature of heating. Figs. 5 : 14—5 : 17 illustrate the type of curve obtained.

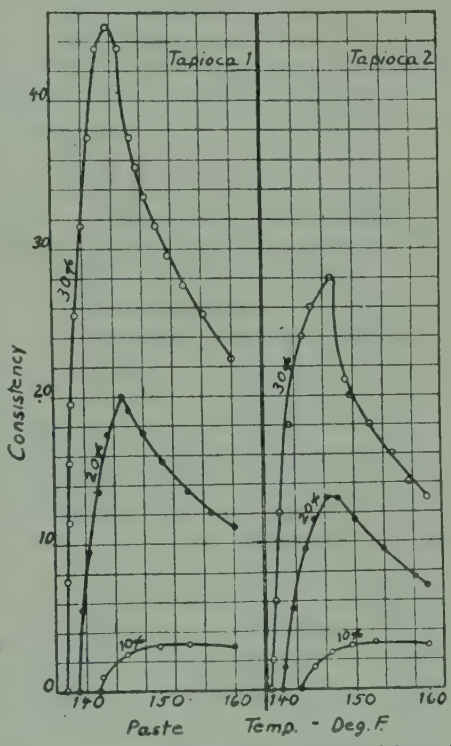

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FIG. 5 : 14.—Consistency Record of tapioca-flour pastes at 10 to 30 per cent. concentration. Caesar's method.

At first the current consumed remains constant until some of the granules begin to gelatinise, when it increases to a maximum and then falls to a minimum during the boiling-period, but again rises as the paste cools down. In Fig. 5 : 14, which shows the effect of concentration of tapioca starch on the viscosity of the paste and illustrates only a part of the complete curve, a notable displacement of the peaks will be observed. These displacements are only small, but they are real and significant. With a concentration of 20 per cent. starch, the swollen granules are closely packed, and the shearing strains imposed on them are therefore high; hence there results a quicker breakdown at a lower temperature than with a 10 or 15 per cent. paste.

Another significant feature is the appearance of a dip in the curves after the peak has been passed, which is especially notice-

able on curves obtained with the higher concentrations of starch. These are probably due to the delayed rupture of the smaller granules, which are the last to swell, offsetting the effect of breaking ing down the structure of the paste brought about by heating and stirring. A quicker falling off in viscosity is noted for the higher concentrations than for the lower, due no doubt to the greater disintegration brought about by the higher internal strains set up in the higher concentrations.

When this method is applied to different starches (Figs. 5 : 14–17) certain similarities are brought out by the curves. The curve for potato (Fig. 5 : 17) resembles that of tapioca, having the same form, and it is seen that wheat, potato and tapioca starches all begin to gelatinise at the same time, but wheat starch gelatinises more slowly than the other two. This may be because the vesicles of potato and tapioca are less resistant to damage than those of wheat starch.

As maize and wheat starch pastes give rather brittle gels on cooling, the curves for these two starches in this region are not the true curves, otherwise the curve for maize starch should cross that of wheat starch and finish well above it. Another factor contributing to this error is the slow rate at which these pastes cool. The cooling-curves for potato, tapioca and sago starches are practically linear, but that for wheat is curved, showing a short-flowing paste.

As the peak values show the swelling power of the starches, Caesar considers that these values can be used to compare different samples of starch. The course of the curves in this case will show the stability to heat and agitation of different samples, factors which may be influenced by the previous history of the starch. One starch may, for example, show a lower peak value than another, but the rest of the curve may indicate that it is more stable to heat and agitation. The swelling action of sodium hydroxide on starch has also been followed by Caesar by this same method.

The author has used a similar method to that of Caesar, only keeping the current in the motor constant and counting the r.p.m. Plotting r.p.m. against temperature gave curves similar in shape to those obtained by Caesar, but inverted. The rate of heating greatly influenced the inflection points in the curves, which were not readily duplicated. Maintaining a steady temperature ($\pm 0 \cdot 25^{\circ}$ C.) for 20 minutes around the gel temperature for the mass of granules, often gave a series of discontinuous drops in the r.p.m., indicating that some granules either took an appreciable time before they started swelling and then gelled rapidly

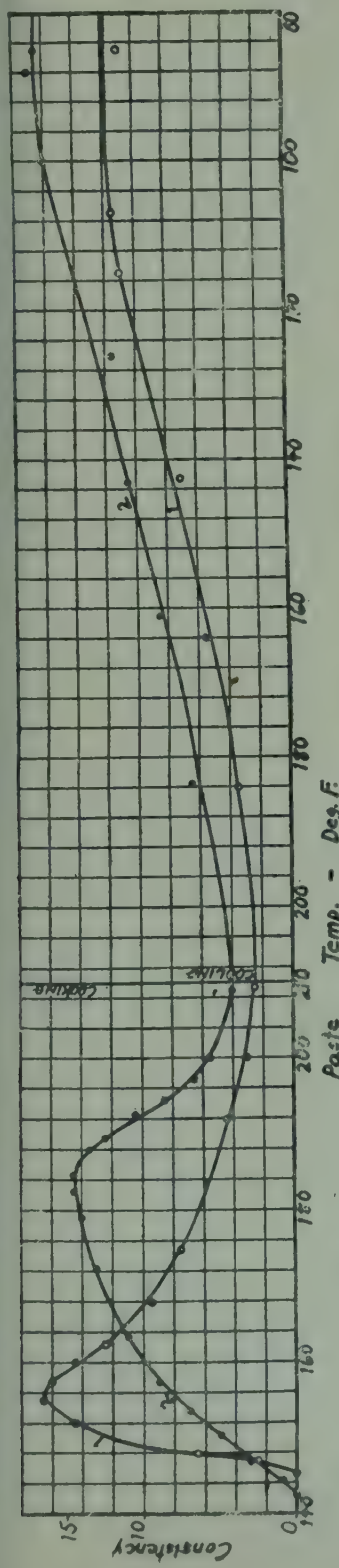

FIG. 5 : 15.—Consistency Record of 20 per cent. wheat (1) and sweet-potato (2) pastes.

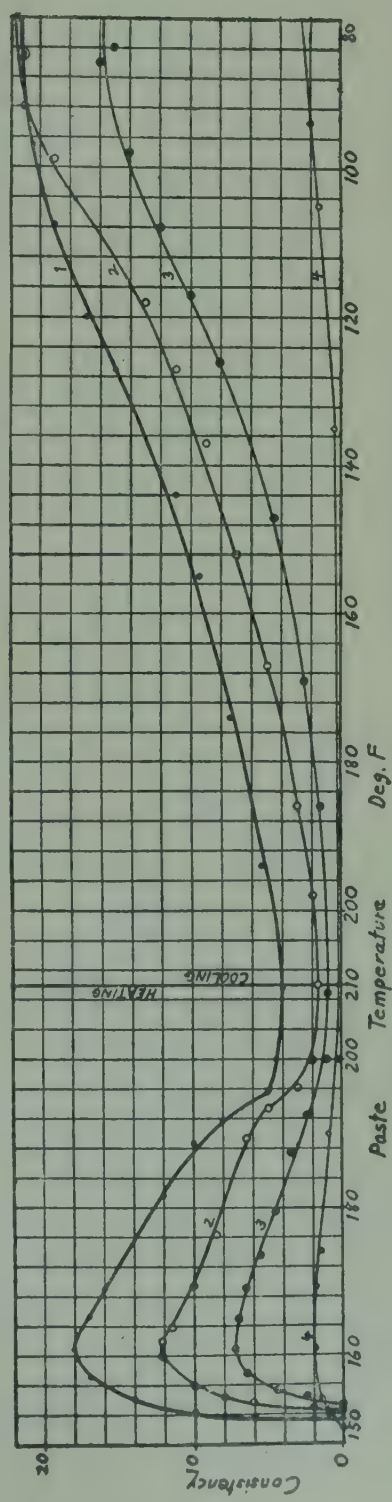

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FIG. 5 : 16.—Consistency Record of 20 per cent. corn-starch pastes.

of that they were coated by the already gelatinised starch, making the free water difficult of access.

It may be noted that H. Herschel and C. Berquist[42] find the Bingham and Green plastometer the best instrument for determining the consistency of starch and dextrin pastes. The rigidity and yield-shear values, as given by this instrument, indicate the 'body', spreading power, and gel-forming power of starch or dextrin preparations, and should be of interest in the textile and adhesive trades. These results are confirmed by other workers.

Recently other workers have described and used an instrument called the amylograph for the study of the gelatinisation of starch. In this instrument the response, which is automatically traced on a chart, is essentially linear to variations in load and the precision appears satisfactory. The influence of the temperature, at which the starch suspension is held prior to commencing heating to bring about gelatinisation, was shown by the fact that with wheat starch suspensions at initial temperatures above 45° C. and gelled in the amylograph the viscosity of the paste was greater than corresponding pastes made from suspensions which were gelled from a lower initial temperature than 45° C.

With this technique a uniform rate of heating is important. As the starch concentration is increased the temperature of transition and the paste temperature at maximum viscosity decreased and the maximum viscosity increased. The relationship between maximum viscosity and the concentration of potato, maize and wheat starches was considered by these workers to imply that the swelling is a first order process, while granule disintegration is a second or higher order process. Addition of wheat gluten to wheat starch suspension was found to give higher viscosity pastes than the same concentration of starch alone. The maximum viscosity of wheat starch pastes decreased in a linear fashion with increase in *p*H value from 5·2-6·8 and also decreased by increasing the maltose value by adding takadiastase. The liquefying effect of α-amylase by corresponding additions to various wheat starches resulted in wide differences in paste viscosities, a fact which may be of importance in the food industry. Gelatinisation with chemical agents was also studied, sodium salicylate being the most, and urea the least, effective. Cold gelatinising agents such as these increase the ability of the starch to swell without granule disintegration. Native, acid- and chlorine-modified starches all show different curve characteristics and the amylograph appears to be a very convenient instrument for investigations on the gelatinisation characteristics of different native and modified

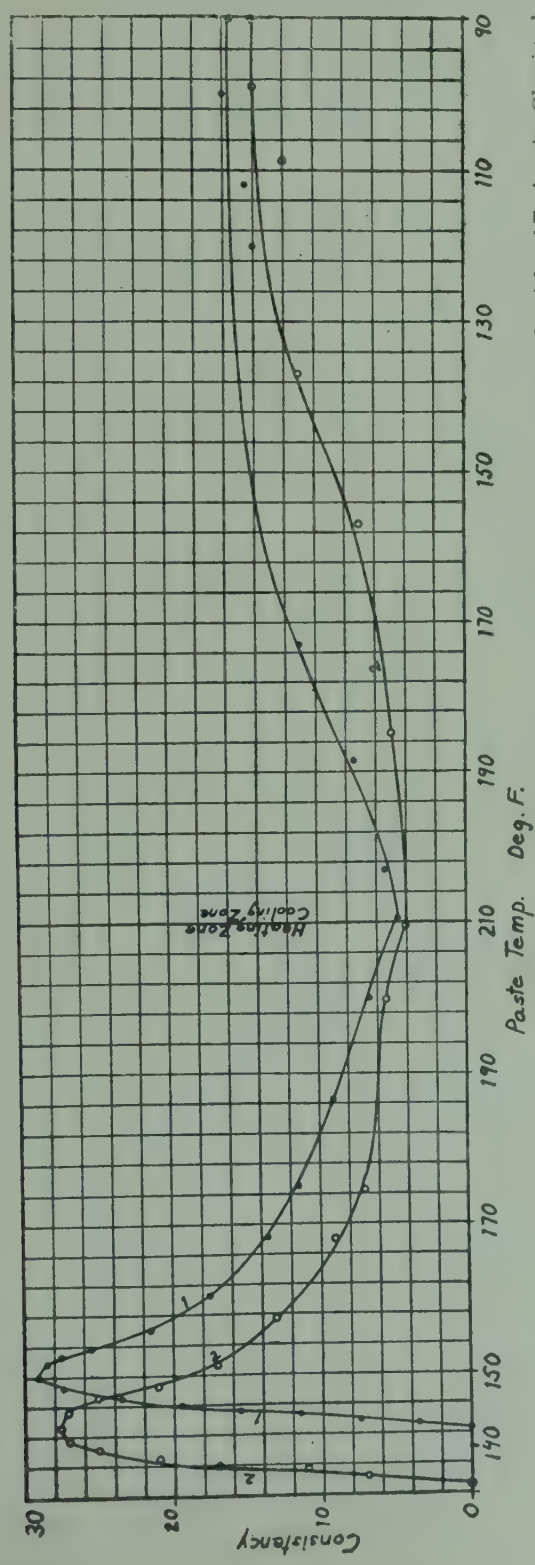

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FIG. 5: 17.—Consistency Record of 20 per cent. potato-starch pastes. 1. Imported. 2. Domestic.

starches and their relative resistance α-amylase and to other starch modifying and degrading agents.

Curves for the gelatinisation of several different starches have been given by W. S. Reich.[21] and the influence of particle size on the viscosity of pastes has been studied by several workers. E. Szego[22] found that the best-quality potato starches contain 1·5-6 per cent. by weight of the granules having a diameter less than 20 μ, and that 43-44 per cent. by weight of the granules have a diameter exceeding 40 μ. On separating the small granules and determining the viscosity of the paste obtained, it was found to be greater than that given by a paste of the larger granules. It was also noted that such pastes had a lower acidity than those obtained from large granules. J. Grüss[23] made similar observations on barley starch, and found the ratio of the viscosity of the paste given by the original starch to that of the small granules to be 1 : 3 approximately, thus agreeing with the amounts of amylopectin present in the samples as determined by this worker, viz. 12·8 and 34·8 per cent. The specific gravity of the large granules was found by these workers to be 1·526, and that of the smallest granules 1·144. J. Janicki[24] confirms E. Szego's finding that the small grains of potato starch yield more viscous pastes than the large grains. He considers that neither the total phosphorus present nor the amount of non-dialysable phosphorus of the starch influences the viscosity of the paste when potato starches from different sources are under consideration. A. Fernbach, however, claims that the smaller grains of starch are richer in phosphorus than the larger granules.[25] Sprockhoff and Parlow[33] are other workers who have found that pastes made from potato starch are more viscous when the granules are very small. They point out, however, that it is essential that the starch should be rapidly manufactured and used soon after making. The inferior tenacity and viscosity of smaller grained starch found in practice is due to the longer treatment required for its preparation and separation. Not only is a more prolonged enzymatic action permitted to take place at its surface but this area is relatively great in the case of the smaller starch granules.

Anker and Geddes[117] find that with wheat starch stored at 9 per cent. moisture content at room temperature a decrease in the maximum viscosity of the paste occurs with increase in storage time whilst Desai[118] finds that high storage temperatures convert high- into low-viscosity potato starch and vice versa for low storage temperatures, observations which both have practical significance, if confirmed.

Other workers[119] have examined the viscosity and microscopical

characteristics of potato and sweet potato starches and find that the varietal and environmental differences are reflected in the viscosity, gelatinisation temperature and granule size and granule size frequency measurements of the starches from various sources. No direct correlation of granule size and the granule size frequencies as they were affected by such differences were found with either the viscosity or the gel temperature: nor were the gelatinisation temperatures and the viscosities directly related. They found that late harvest tends to raise the gel point and decrease the viscosity but the average size of the granules was either smaller or larger depending on the variety. Other workers examining varietal differences as reflected in properties of the starch find that with starches from hard corn and soft corn the former give more viscous pastes than soft corn starches of the same granule size (see, however, R. Kerr, Vol. II). Zika[120] reports that treatment of seed pieces with β-indolyl acetic acid results in increased size of tubers which contain larger starch granules than usual but no viscosity measurements appear to have been made on the starches.

W. A. Richardson and R. S. Higginbotham[67] separated one sample of potato starch into various fractions. Each fraction contains granules of uniform size but varied from other fractions in the mean radius of the granules. They found the phosphorus-content to increase with decreasing mean radius while the copper reducing powers, specific viscosities in 30 per cent. calcium thiocyanate solution, the solubilities of the fractions in 0·01 M. phosphate buffer solution, *p*H 7, are almost identical and the differences in swelling capacity in the same buffer solution are small. It would therefore appear that the average chain-lengths are the same and that the granules have the same structure.

The swelling was much higher in distilled than in salt water and the differences in swelling between the various fractions are not due to differences in the volume/surface area ratio of the granules.[29] As the swelling of wheat starch is unaffected by the above factors it is evident that esterified phosphoric acid affects the swelling to an extent dependent upon the amount present but only when the average chain-length and structure of the granules are the same and no free electrolyte is present.

Janicki also thinks that the major portion of the phosphorus (91·7-99·7 per cent.) is adsorbed on the starch as colloidal phosphates of Ca, Mg, K, Fe and Na, the rest being undoubtedly held in the form of an ester. He considers that the decrease in the viscosity of starch pastes on ageing cannot, therefore, be explained by the saponification of the organic phosphoric ester, but

is induced by changes in the degree of hydration of the starch and of the colloids present. He is thus opposed to Samec's views.

The Effect of Injury to Starch Granules before Gelatinisation on the Properties of the Pastes.—From what has already been said about the lowering of the viscosity of starch pastes by mechanical treatment, owing to the more or less organised structure of the paste first formed being destroyed, it might be expected that severe injury to the outer portions of the starch granules before making the pastes might have some effect on their viscosity, as the swollen granules would not occupy the same space as those of an uninjured starch. Observations on this point show that these expectations are fulfilled.

C. L. Alsberg and E. P. Griffing[26] have noted that over-grinding of flour resulted in the appearance of injured starch granules which disperse in contact with sufficient water. Scheffer[27] notes that such injured granules are easy to detect with the polarising microscope, or by staining them with Congo red and examining under the microscope in the usual manner. L. H. Lampitt and co-workers[76] consider Congo red to be more suitable for this purpose than iodine as the detail is not obscured using the former. They note that after 50 hours' ball-milling the granules of wheat starch did not appear to be injured but Congo red then stained them a clear red. Maquenne[28] has found that the soluble matter from the raw starch when acted upon by amylase rises from 2 per cent. for uninjured starch granules to about 94 per cent. for starch which has been severely ground. However, Alsberg and Griffing[75] find little difference in the rate of formation of reducing substances from badly damaged starch and gelatinised starch when these are subjected to the action of enzymes. W. W. Lepeschkin[37] has made the interesting observation that if starch granules are cut up under cold water swelling does not take place. It would appear possible, therefore, that the heat generated by grinding may bring about some change which assists swelling upon subsequent immersion in water. Lampitt and co-workers,[76] however, suggest that the swollen granules obtained by milling wheat starch for 200 hours and then suspending it in water are substantially in the same state as those swollen by heating in water and that gelatinisation, therefore, depends on the appearance of water-permeability of the granules, which has probably been brought about by some change in structure, and is not due essentially to the action of heat. Definite proof of the absence or presence of an outer membrane on the starch granules, which has indeed been suggested by some workers,[87] and whether

it is protein[87] or hemicellulose as suggested by Ling, would be of interest in this connection. The observed dependence of the gelatinisation temperature on thermal pre-treatment of the granules supports the conclusions of Lampitt and co-workers.

These workers have made an extensive investigation on the various fractions obtained by ball-milling starch. They obtained three fractions which were arbitrarily fixed by their solubility in cold water (the C.W.S.F.), in hot water (the H.W.S.F.) and a fraction insoluble in hot water (H.W.I.F.). When solutions of the cold or the hot water-soluble fractions were dried the resultant material, in each case, showed lower solubilities upon attempting to redissolve them. Samec[77] and also M. Friese *et. al.*[78] have observed the same behaviour with starches, and Hanes and Cattle[79] and Haworth and co-workers[80] with high molecular weight dextrins. The strength of such films appears to decrease with decrease in molecular weight of the film-forming material.[76, 81]

Lampitt, Fuller and Goldenberg find that on ball-milling the X-ray pattern disappears quickly, the organised structure is destroyed and also considerable depolymerisation takes place. The C.W.S.F. increases quite slowly after the X-ray pattern has entirely disappeared and continues to increase, and after 2,000 hours' milling wheat starch is almost completely soluble in cold water. It retains, however, the essential characteristics of starch even when milled for 4,000 hours. These facts point to the conclusion that depolymerisation and destruction of the granule structure are distinct from each other, and from the observations of these workers on the reducing powers of the various fractions it appears that the milling only ruptures the lateral links between the starch chain-molecules but does not induce breakdown of the repeating units.

During the course of milling the rate of formation of the hot-water soluble fraction from the insoluble portion appears to be very similar to the rate of transition of the former into the cold-water soluble fraction. It is possible that, as suggested by Heiniger,[82] some further depolymerisation takes place during the extraction with hot water. The H.W.I.F. has the characteristics of amylopectin, the H.W.S.F. of amylose and the C.W.S.F. of soluble starch. This work is highly important as it shows the ease with which amylopectin is apparently depolymerised to amylose, and agrees with the results of Svedberg,[83] Lamm,[84] Richardson[85] and others,[82, 86] which indicate that the methods of extraction and fractionation used to prepare amylose and amylopectin may determine the relative proportions of these two compounds in any given sample; the greater the severity of the

process the more amylose appears to be obtained. Delffs[51] has utilised the solubility of severely ground starch to prepare solutions of starch.

Advantage has been taken of the fall in the viscosity of pastes from ground starch to obtain the cold-water dispersible starches (see Vol. II.) commercially, and apparently the greater the degree of severity of grinding to which the starch is subjected the better the dispersion obtained in cold water. The viscosity of the pastes made from mechanically injured starch falls as the extent of the injury is increased.[29] If potato starch is ground in a pebble mill, and samples withdrawn from time to time are made into pastes, their viscosity is found to fall in a fairly regular manner, and Katz[61] has suggested that such a ground potato starch might give special technical effects if used for sizing yarns.

Starch granules injured by grinding do not show the black cross when examined with a polarising microscope, but they are still birefringent.[30] Solutions of these preparations in cold water deposit a flocculent precipitate on standing in the same manner as a solution made by boiling untreated starch. After grinding wheat starch for several days in a pebble mill about 60 per cent. of it disperses in cold water, and by successive grinding of the residue and extracting it with cold water practically all the starch can eventually be dispersed. This part of the subject is of especial interest to bakers and millers, and has been studied from this point of view by several workers.

Amylose can be separated from amylopectin by means of the grinding technique, the sample being ground until the viscosity of its solution in cold water is practically constant and the solution dialysed. This yields a gel of amylopectin and a solution of amylose. The point at which the viscosity has fallen to the minimum value is readily reached with the gramineæ starches, but the process is more protracted with others. Woodruff and MacMasters[44] point out that starch prepared from frozen corn sometimes gives lower viscosity in solution than starch from unfrozen corn, and indicate that added chemicals affect maize starch more than wheat starch.

S. Ono[68, 88] has made some interesting observations of the effect of ultrasonic waves on starch paste. The disintegration of potato and wheat starch pastes by waves of 470 and 800 kc. per second was followed by observation of the changes in specific volume and microscopically. Potato starch is affected quicker than wheat starch and the disintegration is greater the higher the frequency used. The reduction in specific volume is comparable with that caused by heating to 150° C. Gases dissolved

in the paste appear to have little effect and Ono considers that the disintegration takes place due to the mechanical action of pulsating bubbles which, by their adiabatic compression, cause a rise in temperature which, in turn, effects hydrolysis of the starch. The frictional forces caused by the vibration of medium-sized particles, and the changes in kinetic energy of the paste granules appear to have no effect in the early stages of the disintegration but some depolymerisation accompanied by oxidation may occur.

W. Seck and G. Fischer have studied the behaviour of starch solutions at high rates of shear and find that after treatment at 70° C. in a colloid mill the viscosity of the pastes fell by 56-98 per cent. (see Chapter 7, Vol. II).

Viscosity and Structure.—The viscosity of colloidal solutions is influenced by the number and, what is important from our point of view, the shape of the colloidal particles.[89] Abnormally high 'specific viscosities' (see below) are characteristic of colloidal solutions having thread-like micelles or macromolecules present. These macromolecules can form an irregular, ever-shifting network, and the effective length of the molecules and therefore the molecular weight will decide the viscosity, those with the highest molecular weight and greatest length exerting a disproportionate effect on the viscosity compared with the effect contributed by a much larger number of those of lowest molecular weight. In starch solutions where a mixture of polymerides is present this state of affairs is particularly true. As the average molecular weight of the polymerides decreases the abnormal specific viscosity becomes less marked owing to the fact that a network resisting flow becomes more and more difficult to form as the length of the chains present decrease. A critical value will be reached below which any further decrease in the molecular weight will cease to have any effect on the viscosity. The increase in reducing power, which is dependent on the terminal aldehydic groups of the starch molecule, will, however, continue steadily with decreasing molecular weight. Thus, providing a homogeneous solution is used, viscosity determinations can be used to characterise sols of starches and dextrins but only when the molecular weight is high and, as Lampitt *et al.*[90] point out, they should prove valuable where the reducing power is unconnected with molecular weight as in oxidised starches[91] and where the terminal aldehyde groups have been destroyed.

From his work on viscosity Staudinger[89, 92–95] concludes that the starch molecule occupied an intermediate shape between the thread-like molecules of cellulose and the spherical ones of

glycogen, and the work of Lampitt, Fuller and Goldenberg (*vide infra*) appears to confirm this conclusion.

Staudinger has found that solutions containing thread-like molecules obey the empirical equation η_r or $1 = K_m CM$ where η_r is the viscosity of the solution relative to that of the solvent (for starch solutions—water; for rubber—benzene, etc.). K_m is a constant dependent on the solvent, C the concentration of the solution and M the molecular weight. Thus $(\eta_r - 1)$ C or η_s/C would be directly proportional to the molecular weight. Staudinger and co-workers,[92, 94, 96–98] Mark and Simha,[99] Hirst and Young,[100] and others[101] find the equation to hold for solutions of starch esters in organic solvents, and Samec[102] has verified it for aqueous solutions of β-amylose and dextrins of high molecular weight which gave practically the same values for K_m, as also did potato and wheat starch acetates in chloroform. K_m was obtained by osmotic pressure data but a number of workers[103–106] have pointed out that this only measures the 'number average', but that the viscosity of a mixture of polymerides of differing molecular weights will depend on the weight-average molecular weight. For the comparison to be strictly valid only solutions containing material of homogeneous molecular weight can be considered. Samec's value of K_m cannot be accepted without further experimental data since his data show that the dextrins and β-amylose he used contained polymerides of varying molecular weight.

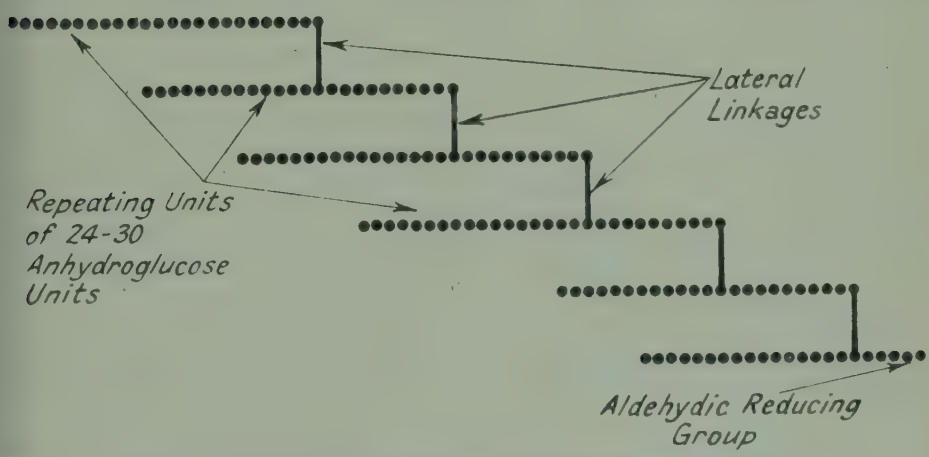
It is therefore necessary to obtain a value of K_m by comparing the viscosity data with that obtained using material of homogeneous molecular weight or by comparison with data obtained by the ultracentrifuge in which the weight-average molecular weight is determined.

In the absence of any other values for K_m for aqueous starch solution Lampitt, Fuller and Goldenberg[90] used the expression η_s/C_g, which is directly proportional to the molecular weight for the data obtained on the viscosities of wheat starch fractions prepared by dry milling. They concluded that the molecular weight of the cold-water soluble fraction (C.W.S.F.) apparently remains constant, whilst that of the hot-water soluble fraction (H.W.S.F.), calculated from both viscosity and reducing power data, starts and continues to fall after 50 hours' milling, and that it obeys Staudinger's equation. Although the values of η_s/C_g are constant for the C.W.S.F., the molecular weight, calculated from reducing power data, falling continuously with continued grinding. With one fraction (300 hours' grinding) the reducing power increased slightly on boiling the solution but the viscosity

was not altered. This illustrates the point made above that the molecules in this case were no longer thread-like[107] and therefore their influence on the viscosity has ceased to be significant.

The spherical macromolecules of glycogen in solution do not obey Staudinger's rule,[93, 95, 108–110] and solutions of the C.W.S.F. appear to behave similarly. The H.W.S.F., on the other hand, appears to behave like the thread-like molecules of cellulose, which follow the rule.[111] These facts are in accordance with Staudinger's conclusion that the starch macromolecule is midway between glycogen and cellulose in its properties.[92–95]

Fig. 5:18[112] shows diagrammatically that 'high-molecular' starches would have a definite thread-like character, i.e. the width is small



Structure of the Starch Macromolecule

FIG. 5:18.

compared with the length, and that the viscosities would follow Staudinger's rule. With the C.W.S.F., however, as the macromolecules have a much shorter average length,[76] the width becomes comparable to the length, and the Staudinger rule ceases to hold as the molecules are no longer thread-like.

Fig. 5 : 19 shows why an aqueous solution of a high molecular dextrin can follow Staudinger's rule.[102] The starch macromolecule is diagrammatically represented as having 11 repeating units, each being a chain of 30 anhydroglucose groups, each unit being connected by a lateral link. In the diagram of the dextrin (a 'high-molecular' dextrin) the repeating units have been shortened from 30 to 12 anhydroglucose units but the lateral linkages are intact. Its molecular weight would thus be about 40 per cent. that of the starch, but it would be more thread-like in nature than starch and would be expected to show a similar

relationship between the reducing power and the viscosity of its solutions as has been found for the H.W.S.F. starches by Lampitt

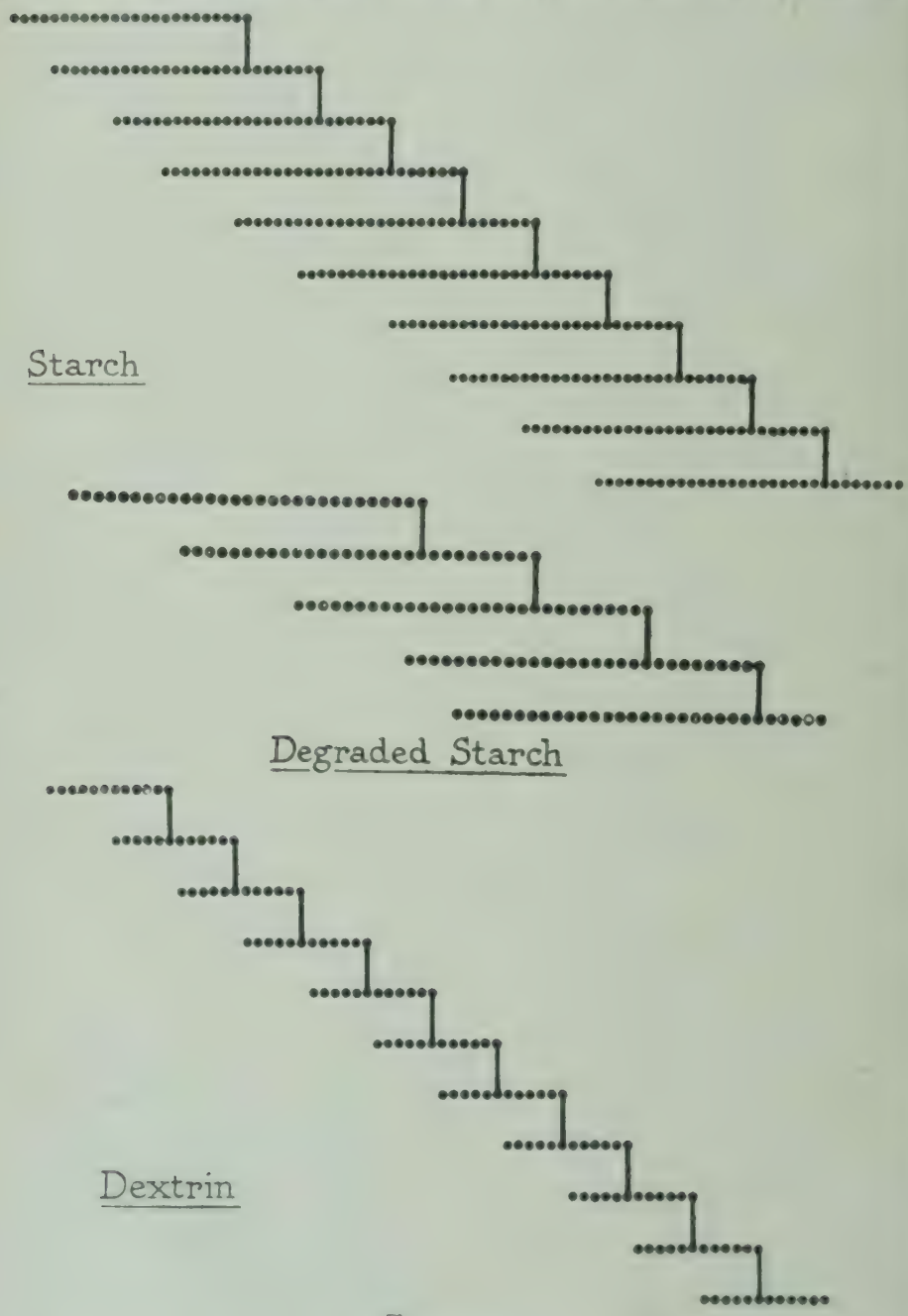


FIG. 5:19.

et al. If the lateral linkages are broken, as shown in the last diagram, then the breadth becomes comparable with the length, and Staudinger's rule is not followed. The inverse relationship,

observed by Lampitt and co-workers, between the viscosity of the H.W.S. starch fractions and their reducing power has also been noted by other workers for starches[113] and yellow dextrins.[114] Other workers have shown that viscosity is a function of the molecular weight, or of the properties connected with it in the case of starch.

The Rigidity of Starch Pastes.—The properties of elasticity and rigidity are very closely related (for definition see E. C. Bingham[52]), and in the case of starch pastes the determination of these values gives interesting results. S. M. Neale[53] has measured the elasticity of air-dried films of starch. F. T. Peirce[34] had applied some sensitive and non-destructive tests of rigidity to films of potato starch, and such tests are also applicable to the testing of yarns and fabrics so as to determine the effect of sizes or finishing processes. He finds that there is a rectilinear relationship between the change in torsional rigidity and the humidity. The rate of change of flexural rigidity is nearly the same, from 30-80 per cent. R.H., whilst the effect of humidity on Young's modulus is expressed by sigmoid curves. The torsional and flexural rigidities vary together over most of the range, 34-65 per cent. R.H., and he concludes that the test of resistance to twisting provides adequate evidence of the effects of moisture on the elastic properties generally. The amount of swelling is different in different directions, and the thickness of the film increases at a rate 70 per cent. greater than the length. This tends to show that the water goes between rather than into the layer parallel with the surface. C. E. G. Porst and M. Moskowitz[43] attempted to measure the 'yield shear value' by extrapolation of flow-shear curves but obtained indefinite results owing to the gradual slope of the curves obtained. They found the results are considerably influenced by the temperature at which the pastes are prepared, but that the plastometer is of value as an aid to matching submitted products. F. D. Farrow, G. M. Lowe and S. M. Neale[54] observed the flow in starch pastes at rates of shear below Bingham's theoretical yield value. Farrow and Lowe[73] had previously shown that the rate of flow of starch pastes, when driven through a capillary tube, is not proportional to the pressure but increases more rapidly than the pressure increases. Newton's postulate describing the flow of most liquids at low speeds was found not to hold for starch pastes by F. D. Farrow, G. M. Lowe and S. M. Neale.[54] The equation which the latter workers have introduced contains more than one constant characteristic of the liquid and appears to describe the flow of starch pastes better than any other two-constant

equation despite the fact that it does not hold, even approximately, over a wide range.

These workers point out that since in most viscometers the shearing stress is different at different points in the liquid the distribution of shear will be different from that shown by liquids obeying Newton's postulate. Their expression is evolved in terms of stress and strain which allow the measurements to be expressed independent of the type of viscometer and allow comparison of results of different workers using different types of viscometers.

The work was carried out over a total range of shear of 300,000-fold, and they found no evidence of discontinuity of the flow-pressure curve such as would be required to satisfy the yield-value hypothesis of Bingham that no flow takes place below a certain value of the applied stress. The stress-shear curve for starch pastes either passes through or terminates, near to the origin.

U. Ebbecke and R. Haubrich[74] find a great increase in the viscosity of starch solutions when pressures of 400-800 atmospheres are employed.

S. Woodruff and M. M. MacMasters[60] measured the relative viscosity and gel-strength of starch pastes from different varieties of corn, or the same variety of corn treated in different ways, and found viscosity differences were very small compared with differences in gel-strength. The gel-strength fluctuated widely with the variety of corn from which the starch was made and with the growing conditions of the corn. Commercial starches gave weaker gels than samples carefully prepared in the laboratory. These workers used the Tarr and Baker[55] jelly tester to determine gel-strength, whilst a Stormer viscometer was used for viscosity determinations (see Chapter 19, Vol. II). Champion White Pearl, Sutton White Dent, Pioneer Hi-Bred 305A, Reid Yellow Dent and a Reid starch gave gels in that order of strength, the latter giving a gel as weak as the commercial starch samples. Corn grown under poor conditions or frozen in the ear gave weak gels, the viscosity, however, was affected but little in comparison to gel-strength.

As the gel-strength of certain starches for use in foodstuff industry, e.g. for blanc-mange powders, etc., or in the adhesive industry is important, this work emphasises the importance of the determination of gel-strength, which is a better guide than viscosity data for the selection of starches to be used for certain purposes.

C. M. McDowell and F. Usher[56] measured the rigidity of non-aqueous suspension of raw starch, but their work is of no

interest to us here. G. P. Arcay and Etienne[57] include starch in the substances whose gels they examined for the presence of rigidity. It should be pointed out that consistency as measured by Caesar (*v.s.*) is partly conditioned by viscosity, plasticity, thixotropy and rigidity. B. Brimhall and R. M. Hixon[58] have modified a MacMichael viscometer[66] into an apparatus resembling that of Schwedoff,[59] and they obtained no correlation between the viscosity and the rigidity curves, which indicates that these values measure different properties of the pastes ; and they consider that rigidity is dependent upon the condition of the granule membrane.

These workers[115] have more recently introduced a gelometer for measuring breaking strength and elasticity of starch gels which embodies the following principles : Suction is used to deform the gel, the volume of deformation being measured by hydrostatic means. ' Skin effects ' and the influence of the containing walls are both eliminated and the deforming force changes uniformly and at a constant rate. This apparatus is suitable for starch concentrations of 6·5 to 8·5 per cent., but for lower concentrations the modified MacMichael instrument mentioned above is preferable.

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Chapter 6

THE STARCH FRACTIONS

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Introduction.—The most perplexing and disputed problem in the long history of starch chemistry has been that of the heterogeneity of starch, whether it is composed of a single chemical entity or whether it contains two or more different polysaccharides. The literature records a century of controversy over this subject, with opinion alternately favouring a single starch component and a plurality of components. Within the past fifteen years, convincing evidence has finally been presented that the common starches contain two different fractions, and very substantial progress has been made in elucidating their individual structures. Improved techniques have been developed for the isolation and purification of authentic fractions, and quantitative criteria have been devised to describe and differentiate these components. In addition, some understanding has been gained of the biochemical reactions which are responsible for the formation of the starch fractions in the plant organism. In retrospection, it appears that this progress has been geared directly to the development of modern physico-chemical concepts of high polymers. The behaviour of starch could not be adequately interpreted in terms of molecular structure either by classical sugar chemistry or by the older theories of colloidal systems.

Virtually all methods of starch fractionation have been based on differential solubility of the fractions in various aqueous media. Either the more soluble component has been leached out of the total starch substance, or the less soluble component has been precipitated from a starch solution. It is now recognised that the solubility behaviour and solution stability of such high polymeric substances do not follow the simple pattern of crystalloids, since the degree of molecular aggregation profoundly influences solubility. There is no sharp demarcation between insoluble and dissolved states, but rather a continuous spectrum of ' solvation ' levels, depending on the extent of disaggregation. In this sense, the native starch granule is insoluble because of its high degree of

intermolecular organisation ; solution can only be effected through a progressive process of hydration, swelling, and disintegration of the swollen granule. Conversely, a starch solution is a highly unstable system, undergoing spontaneous and progressive aggregation to a less soluble state. These physical changes radically alter such characteristics as the viscosity, enzyme susceptibility and ease of esterification of the starch substance. For example, it has been claimed that the insolubilisation of a starch solution can be detected by increased resistance toward enzyme action long before any change is apparent to the eye.

In studying the composition and constitution of starch, extreme care must be exercised to avoid even the slightest chemical degradation, such as might occur through hydrolysis or oxidation or by too rigorous drying. The most minor molecular degradation (undetectable by any ordinary chemical means) may cause inordinately large changes in the physical properties of the starch substance. The investigator must also be discriminating in his choice of raw starch, since many of the presumably unmodified starches of commerce have been subjected to some sort of chemical treatment. The writer recalls the apparent presence of three fractions in a particular type of corn starch, eventually traced to the fact that the starch had been prepared from alkali-steeped corn.

Thus, the principal factors which have complicated the fractionation of starch are : (1) the physical instability of starch systems ; (2) the difficulty of avoiding minor degradative changes ; and (3) the absence of suitable criteria for differentiating and characterising the starch fractions. In addition, the literature has been hopelessly confused by nomenclature, the frequent coining of new terms without adequate definition, and the indiscriminate use of old terms, often in a reversed sense. It is frequently impossible to decide the identity of some starch fraction either from its assigned name or even from a description of its preparation and properties.

Earlier Fractionation Methods.—The earliest recognition of the presence of several constituent substances in starch is usually ascribed to the microscopist Leeuwenhoek[1] in 1716. When starch was heated in water, he observed that the integument or hull of the granule remained undissolved. Since similar integuments were observed in the excrement of birds, Leeuwenhoek differentiated between the outer indigestible hull of the starch granule and the inner 'nutritive substance'. No further reference to starch components can be found until the early part of the nineteenth century, when the subject was studied by de Saussure[2]

(1819) and by Raspail[3] (1825). These writers describe a water-soluble component (' amidine ') which stained blue with iodine, and an insoluble cellulosic material (' ligneux amylacée ') which appeared to constitute the outer sheath of the granule. These studies were extended by Guibourt[4] (1829) and particularly by Guérin-Varry.[5] Based on the solubility behaviour of swollen and of crushed granules, these investigators defined three components of starch, a fraction soluble in cold water and giving a blue iodine colour (' amidine '), a second component (' amidine soluble ') which was soluble only in hot water but was held in solution by the amidine, and an insoluble fraction which constituted the outer hull of the granule (' amidine tegumentaire '). The relative amounts of these components are given by Guérin-Varry as 59 per cent., 38 per cent., and 3 per cent., respectively.

The tri-component theory has never been widely accepted, though it recurs at frequent intervals. The extensive investigations of Naegeli[6] served to focus attention on a bi-component concept. Naegeli (who may be considered as the father of starch chemistry) was primarily concerned with the botanical structure and growth of the starch granule. He pictured its growth as due to deposition of carbohydrate within the granule (' intussusception '), rather than by accretion on the outside (' apposition '). The granule consisted of two distinct substances, an older outer layer of ' starch cellulose ' (which Naegeli considered identical with wood cellulose), and an inner soluble substance which he termed ' granulose '. The former amounted to approximately one-eighth of the total starch, and was obtained as the difficultly soluble residue which remained after prolonged digestion of ungelatinised starch with saliva. While iodine imparted little colour to starch cellulose, it gave a blue colour with granulose. Von Mohl[7] opposed the concept that starch contained a cellulosic substance, pointing out that wood cellulose was far less soluble in acid or alkali than Naegeli's starch cellulose; he therefore proposed the name ' farinose ' for the latter material. Von Mohl likewise claimed that iodine afforded no differentiation between granulose and farinose, since red or blue colorations could be obtained with either substance, depending on the state of hydration. Naegeli later modified his methods and viewpoints.[8] In place of saliva, he subjected raw starch to the prolonged action of cold dilute acid, after which the granulose was leached out with water, leaving the skeletal amylocellulose (incidentally, the latter was the source of Naegeli's crystalline amylodextrin). However, Naegeli suggested that both granulose and amylocellulose merely represented various physical modi-

fications of the starch substance, characterised by differing solubilities and iodine reactions. These modifications appeared to be interchangeable, one form passing into the other through a series of transition stages. It might be remarked that this concept has recurred persistently until the last decade. Only recently has it been finally established that the starch fractions are not merely physical modifications of one another, but rather that they are distinctly different substances in a chemical sense.

By treating a 5 per cent. potato starch paste with malt extract, Brown and Heron[9] isolated amylocellulose as a filtrable precipitate, amounting to 5 per cent. of the starch. However, much lower yields were obtained if lower starch concentrations were employed, or if the starch was first treated with dilute alkali. Consequently, they questioned whether amylocellulose had any real existence in the granule.

A. Meyer regarded the starch granule as a spherocrystal, built up by the accretion of successive growth lamellae of different densities.[10] He concluded that normal (i.e. non-waxy) starches contain only amylose and a small amount of amylodextrin.[11] However, amylose might occur in two crystalline modifications, a difficultly soluble anhydride form which he termed α-amylose, and a readily soluble hydrated β-amylose. These correspond respectively to the starch cellulose and granulose of Naegeli. α-Amylose was isolated as the insoluble residue on treating pasted starch with malt extract, or by warming starch with dilute acid. The amounts were small, 0·6 per cent. from potato starch, 0·9 from rice, 1 per cent. from corn, and 2·5 per cent. from arrowroot. Meyer visualised the starch granule as composed of crystals of α-amylose, β-amylose and amylodextrin, arranged as radial trichites. Growth periodicity gave rise to concentric shells. When starch was treated with iodine, some lamellae stained red and some blue, depending on the predominance of amylodextrin trichites or amylose trichites (either in α or β form) in the particular lamella. When an aqueous starch suspension was heated to the gelatinisation temperature, it was presumed that the β-amylose crystals dissolved to give an amylose solution. The crystals of α-amylose persisted until a temperature of 138° C. was reached, when they likewise dissolved to give a solution of amylose. Thus Meyer recognised α- and β-amyloses only in the solid crystalline state, while amylose itself could only exist as a solution.

It is difficult to evaluate the work of Naegeli and of A. Meyer on the basis of our present knowledge of starch. They were both interested primarily in the botanical aspects of starch, its mode of

deposition in the plant, the significance of the striae in the granule, and the mechanical organisation of the granule. It must be admitted that in the past fifty years we have not progressed materially in our understanding of these particular problems. Meyer was not concerned so much with the chemical differences between the starch fractions, though he undoubtedly recognised that such differences did exist. This is particularly apparent in his studies on waxy sorghum and rice starches,[12] which he characterised as chiefly amylodextrin by reason of their red iodine coloration. He employed two criteria by which to differentiate between the granule components, iodine colour and solubility. By the first means, he distinguished between red-staining amylodextrin and blue-staining amylose; by the second criterion, α-amylose was difficultly soluble while β-amylose was readily soluble. These two criteria did not coincide because Meyer did not have the means to effect a sharp separation between the fractions and because his methods of separation (viz. digestion with saliva or with acid) caused considerable degradation to give soluble red-staining products.

The first serious attempts to isolate and study the starch fractions must be credited to Maquenne and Roux. By allowing starch pastes or autoclaved starch sols to stand at room temperature, an insoluble material deposited which Maquenne[13] designated first as amylocellulose and later as amylose. In its precipitated state, this product gave little or no colour with iodine. However, if dissolved by heating in water to 150° C., the resultant solution gave a pure blue iodine colour, more intense than that from an equal amount of starch. When amylose was dissolved in water at 150° C. and the solution allowed to cool, the material re-precipitated as 'artificial starch'. This process of solution and precipitation could be repeated indefinitely. Maquenne and Roux[14] employed the term 'retrogradation' to designate this spontaneous insolubilisation of amylose. It is interesting to note that this term had been introduced previously by Lindet[15] to describe the changes which occur in the starch substance of bread during staling.

According to Maquenne and Roux, malt conversion of amylose gave a substantially theoretical yield of maltose (i.e. 96-98 per cent.). Erroneously, amylopectin was believed to be completely resistant toward malt extract. Therefore, since starch gave 80-82 per cent. of maltose, the unconverted 18-20 per cent. was considered to be amylopectin. Comparative iodine colorations appeared to confirm this ratio, since the depth of colour of the starch-iodine complex corresponded to 79 per cent. of blue-

staining amylose and 21 per cent. of colourless amylopectin. Actually, Maquenne and Roux did not isolate amylopectin; its presence was merely inferred to explain the viscous and mucilaginous character of starch pastes (viz. the pectinous principle of starch). Their methods of fractionation were extremely crude, since retrogradation of the amylose necessarily entrained a considerable portion of the amylopectin. Also, their malt extract undoubtedly contained both dextrinogenic and saccharifying amylases, and characterisation of the fractions by such means was hardly critical. Despite the inadequacies of their methods and interpretation, Maquenne and Roux were the first to recognise the existence of two separate chemical entities in starch. This initiated the study of starch from a chemical rather than a botanical viewpoint.

Other and more recent investigators have attempted to effect a separation of the starch components by preferential enzymolysis. Ling and Nanji[16] claimed that amylose was completely converted to maltose by barley diastase, while amylopectin was scarcely affected, thus affording a means for isolating the latter material. Schryver and Thomas[17] treated various starch pastes with takadiastase, dissolved the resulting insoluble residue in 4 per cent. sodium hydroxide and acidified with acetic acid. The cereal starches gave as much as 4 per cent. of insoluble floc which the authors termed 'hemicellulose'. The tuber starches gave no appreciable residue on treatment with takadiastase. Similarly, Ling and Nanji[18] converted starch pastes with barley diastase to give an insoluble floc of 'amylohemicellulose'. This amounted to 7-8 per cent. from wheat starch and 15 per cent. from rice starch; potato and tapioca starches gave no significant amount of insoluble material. Ling and Nanji therefore claimed that the common cereal starches contained three carbohydrate constituents (amylose, amylopectin and amylohemicellulose), while the root and tuber starches contained only two (amylose and amylopectin).

There are several objections to these enzymatic methods. In the first place, it is now known that amylases attack all components of the starch, and that any products isolated are necessarily degraded. Secondly, the extent and the products of an enzyme conversion are influenced by the physical heterogeneity of the system, the presence of retrograded material and resistant granule structures. Thirdly, as will be shown later, the so-called hemicellulose and amylohemicellulose are undoubtedly caused by the presence of such impurities as fatty acids in the starch.

Gatin-Gruzewska[19] employed a system of leaching, whereby

potato starch was swollen in 1 per cent. sodium hydroxide solution, allowing the interior amylose to pass into solution. The mixture was then neutralised with acetic acid which precipitated the empty granule sacs (amylopectin). This latter material constituted 40-45 per cent. of potato starch, gave a violet colour with iodine, did not retrograde, and was therefore likened to glycogen. The soluble extract of amylose retrograded rapidly. Addition of a small amount of alcohol precipitated the amylose as rods or spheres, a behaviour which was considered similar to that of inulin.

Maquenne and Roux, and likewise Gatin-Gruzewska, apparently considered starch to be a mere physical mixture of two entirely unrelated chemical substances, as dissimilar as inulin and glycogen. In seeking to characterise the fractions in terms of specific solubility and retrogradation, they minimised the physical instability of the whole starch substance. In contrast, Fouard[20] claimed that starch could be completely dissolved to give a perfect solution but that this system was highly unstable, tending to revert spontaneously to an insoluble state, especially in the presence of acid. Thus, when a starch solution was ultra-filtered through a collodion membrane and the insoluble residue repeatedly redissolved and re-filtered, there appeared to be no limiting minimum amount of insoluble material. Fouard therefore maintained that starch was not a mixture of different substances, but that it represented a single chemical substance capable of undergoing a reversible physical aggregation to an insoluble state. The amount of insoluble material was determined by the particular temperature, concentration and acidity; this explained the wide variation in the reported composition of starch.

Tanret[21] effected a separation of the starch fractions by gelatinising one part of starch in a thousand parts of boiling water. If the water was maintained at the boiling temperature for only a few moments, the amylose was presumably leached from the swollen granules without dissolving the amylopectin. The swollen granules were separated by sedimentation and extracted two additional times with boiling water. The amount of amylose dissolved in the supernatant solution amounted to 30 per cent. from corn starch, 27 per cent. from potato starch, and 32 per cent. from wheat starch. Tanret devised a unique method to remove traces of amylose from the insoluble amylopectin residue. The latter was dissolved either by prolonged boiling or by heating in a sealed tube at 130° C., the solution cooled and then treated with cotton to adsorb the residual amylose. The resulting amylopectin solution gave a violet or red colour with iodine. Similarly,

when a starch solution was repeatedly treated with cotton, it produced a red iodine coloration. Tanret considered this as proof that starch contained amylose and amylopectin as separate entities.

Ling and Nanji[16] modified the methods of Maquenne and Roux, by operating on potato starch pastes which had first been frozen and then thawed. The resulting cottony mass was repeatedly extracted with water at a maximum temperature of 60° C., to avoid excessive swelling and peptisation of the amylopectin. As a further improvement, they claimed that extraction with 0·5-1 per cent. sodium carbonate solution leached out the amylose more readily than did plain water. No yields were given. As a refinement of these aqueous leaching methods, Mabel Baldwin[22] pasted potato starch at a temperature (63° C.) just sufficient to swell all the granules. This paste was quickly frozen, then thawed and filtered. The insoluble residue was repeatedly extracted with water at 55-60° C., and these solubles combined with the original filtrate. The total yield of soluble extract (the so-called β-amylose) from potato starch was 15·6 per cent. This fraction was characterised by its intense blue colour with iodine, its low phosphorus content and its pronounced retrogradation tendencies. Correspondingly, the residue of swollen granules (the α-amylose) possessed a relatively high phosphorus content and showed no inclination to retrograde. After treating the residual α-amylose with cotton to remove blue-staining material (according to the method of Tanret), it gave a red-violet colour with iodine. Considerable importance was attached to the change in specific rotation with temperature, as a means of characterising these fractions. The α-amylose appeared to have a temperature coefficient of rotation almost ten times that of the β-amylose.

The studies of Samec and his co-workers[23] have shown that the phosphorus content of certain starches is intimately related to the starch fractions. Fernbach[24] had previously observed that potato starch contains phosphate which cannot be removed by washing with water or with dilute acid. According to Gatin-Gruzewska,[25] electrophoresis of an amylopectin solution causes the latter substance to migrate toward the anode. Samec and Haerdtl,[26] and Samec and Anka Mayer[27] attributed the polarity of potato amylopectin to the presence of phosphate ester groups, in an amount corresponding to 0·08 per cent. phosphorus. Since the amylose component is virtually phosphorus-free, these investigators employed electrophoresis for effecting a starch fractionation. When a 2 per cent. solution of potato starch (prepared by autoclaving for one hour at 120° C.) was electro-sedimented,

the amylopectin (Samec's ' erythroamylose ') deposited as a slimy paste on the anodic membrane, leaving the amylose (Samec's ' amyloamylose ') in the supernatant solution. The yields of amylopectin from potato, wheat, corn and rice starches were 74 per cent., 63 per cent., 45 per cent. and 38 per cent., respectively. Amylopectin was characterised by its electrical polarity, the presence of esterified phosphate and its red iodine colour, while the amylose was electrically neutral, contained little or no phosphorus and gave a blue colour with iodine. Samec attributed many of the outstanding differences between the fractions to the presence of this phosphate. The amylopectin was considered as an ' amylophosphoric acid ', capable of forming salts or binding proteins. The high viscosity of this component was attributed to ionisation and solvation of the phosphate groups. When the phosphate was stripped from the amylopectin by prolonged autoclaving, there was a concurrent decrease in viscosity and in electrical mobility. Conversely, these latter properties could be enhanced through the introduction of phosphate groups by esterification with phosphorus oxychloride.[28] Thus the paste-forming qualities of starch were attributed by Samec to the amylopectin component, and more specifically to the status of the latter as an amylophosphoric acid.

The methods employed by Samec to effect a fractionation undoubtedly occasioned a certain degree of hydrolytic degradation. Unless starch pastes are maintained within a very narrow *p*H range (i.e. 5·9-6·3), autoclaving at elevated temperatures will cause hydrolysis of glucosidic linkages. Also, electrophoresis of starch solutions may produce local high acidities, since the dialysing membranes appear to act as floating electrodes. Electrosedimentation is at best a slow process and considerable retrogradation of the starch substance occurs. Despite these inherent difficulties, Samec and his co-workers must be credited with two major contributions to starch chemistry, the development of the unique electrophoretic method of fractionation and the differentiation of the fractions on the basis of phosphorus content. In recent years, the significance of this combined phosphate has been somewhat overlooked. However, it now appears that the enzymatic synthesis of starch in the plant is accomplished through the medium of glucose-1-phosphate. The presence of phosphate in one component of the starch therefore assumes new importance, from the structural as well as the botanical standpoint (see Chapter 2).

Taylor and his co-workers applied Samec's electrophoretic technique to the cereal starches. Taylor and Nelson[29] had ob-

served that these starches, while low in phosphorus, contained a significant amount (0·6-0·8 per cent.) of fatty acid which could not be removed by extraction with the usual fat solvents (e.g. carbon tetrachloride, petroleum ether, ethyl ether). Since these fatty acids were liberated only when the starch was partially hydrolysed, it was presumed that they were esterified with the carbohydrate, having a status similar to that of the phosphorus in potato starch. In order to dissolve corn starch without autoclaving at elevated temperatures, Taylor and Iddles[30] treated the starch with alcoholic hydrogen chloride, then gelatinised this product in aqueous ammonium thiocyanate solution and recovered the starch substance by alcohol precipitation. This treatment gave a water-soluble starchy product containing most of the lipid material of the original corn starch. When this was dissolved in water and subjected to electrophoresis, a minor portion of the starch (14-16 per cent.) migrated as an insoluble floc. Similar yields of insoluble material were obtained when solutions of soluble corn or rice starch were ultra-filtered through collodion membranes. This fraction (Taylor's 'α-amylose') was high in lipid content, and its insolubility and polarity were therefore attributed to the presence of esterified fatty acid. The major fraction of the starch (Taylor's 'β-amylose') contained no significant amount of fatty acid and was therefore water-soluble and non-polar. Taylor and Beckmann[31] later employed corn starch which had been rendered water-soluble by Alsberg's method of prolonged dry-grinding in a pebble mill.[32] Electro-sedimentation of solutions of this ball-milled starch gave fractions which were similar in amount, solubility and lipid content to those obtained from acid-solubilised starch.

Prior to the investigations of Samec and of Taylor, most fractionations had been effected by preferential solution methods, operating on swollen starch pastes and dissolving the more soluble component by leaching or by enzymatic methods. Both Samec and Taylor endeavoured to dissolve the entire starch substance and then to separate the less soluble component from the resulting solution. Alsberg[33] had stressed the fact that a boiled starch paste does not represent a homogeneous solution, but rather a suspension of greatly swollen but relatively intact granules. The viscosity of such a paste was ascribed to mechanical jostling of swollen elastic granules. Taylor[34] maintained that these swollen granules must be completely dispersed before a satisfactory fractionation could be achieved; otherwise, the swollen granules would be arbitrarily included with the less soluble fraction. Unfortunately, the methods employed by Taylor to disintegrate

granule structure (viz. ball-milling or pre-treatment with alcoholic hydrogen chloride) produced marked chemical degradation of the starch, as indicated by the rise in 'alkali labile value'.[35] Also, it is now known that the fatty acids of the cereal starches are merely adsorbed on the starch and not esterified with the carbohydrate.[36, 37] Taylor undoubtedly obtained a partial fractionation by electrophoresis, through the selective adsorption of this free fatty acid on one fraction of the starch. While Samec's amylopectin electro-migrated because of the polarity imparted by its phosphorus content, in the case of corn, wheat and rice starches it appears to be the amylose fraction (Taylor's α-amylose) which exhibited electrical mobility through adsorption of fatty acid. Consequently, both Taylor and Alsberg considered α-amylose to be the equivalent of amylopectin.

Pacsu and Mullen[38] have elaborated on Tanret's method of fractionation by selective adsorption on cotton. When a cold 1 per cent. corn starch paste was treated with cotton, the blue-staining component (β-amylose) was preferentially adsorbed, leaving the α-amylose in solution. The latter was characterised by its relatively high phosphorus content (0·02 per cent.) and by its purple coloration with iodine. The β-amylose could be stripped from the cotton by elution with hot water. It gave a deep blue colour with iodine, contained no phosphorus, and exhibited pronounced retrogradation tendencies.

Structure of the Starch Fractions.—Somewhat illogically, the structure of the individual starch fractions was established before the authentic fractions were actually isolated in pure state. The present concept of starch and its fractions as polymeric chains of α-1, 4-glucopyranoside units had its origin in cellulose chemistry. From X-ray studies, Herzog and Jancke[39] and Sponsler[40] concluded that starch possessed a crystalline space lattice similar to that of cellulose. K. H. Meyer and his associates[41] suggested that such substances as cellulose, starch and chitin were composed of long polymeric 'main valence chains' of glucose units, associated laterally by secondary valence or 'molcohesion' forces. Cellulose was pictured as an extended chain, starch as a crumpled or zig-zag chain due to its α-glucosidic linkages.

The classical methylation studies of Haworth, Hirst and their associates[42] presented strong evidence for a chain-like structure, since hydrolysis of trimethyl starch gave 4·5 per cent. of 2, 3, 4, 6-tetramethylglucose, derived from the non-aldehydic terminal glucose of the starch chain. This corresponded to a chain length of 25 to 30 glucose units. Haworth, Hirst and Woolgar[43] determined a chain length of 26 to 29 units for waxy maize starch,

claimed to be composed entirely of amylopectin. Early methylation studies provided no differentiation between the fractions, probably because the existing methods of fractionation were very crude. Hirst, Plant and Wilkinson[44] separated potato starch by the method of Ling and Nanji, obtaining 17 per cent. of soluble amylose. Methylation analyses of the resulting amylose and amylopectin gave identical values, corresponding to 23 to 24 glucose units. Behaviour of the acetylated or methylated potato starch fractions in chloroform or acetone solution paralleled the characteristics of the parent fractions in aqueous medium, viz. amylose and its derivatives were more soluble and possessed lower viscosity than amylopectin. It was concluded that the fractions were of identical chain length, differing only in their state of aggregation. Thus, amylose was regarded as a highly hydrated substance, tending to retrograde to give interlocked aggregates of amylopectin.

These estimates of the chain length of starch were challenged on both physical and chemical grounds. From osmotic pressure measurements on methylated starch in chloroform solution, Carter and Record[45] derived a molecular weight corresponding to 630 glucose units, as compared with a value of 28 units by methylation assay. In contrast, methylated inulin gave a chain length of 31 hexose units by both osmotic pressure and methylation methods. Richardson, Higginbotham and Farrow[46] observed that the copper-reducing values of various unmodified starches corresponded to average chain lengths of 460 to 1,470 glucose units. They attributed the low values by methylation analysis to hydrolytic degradation during methylation. Richardson and Farrow likewise fractionated corn, wheat and sago starches by a process of pasting, freezing, thawing and leaching. The copper reducing values of the soluble amyloses corresponded to chain lengths of 150 to 170 glucose units; the reducing values of the residual amylopectins were lower than the values for the corresponding whole starches. These investigators concluded that the difference between the fractions was one of chain length, the amylopectin comprising the longer chain molecules.

Staudinger and Husemann[47] finally reconciled these conflicting viewpoints by suggesting a branched-chain structure for starch. Their proposal was based on a comparative study of the molecular weights (from osmotic pressure) and solution viscosities of the acetates and nitrates of starch and of various acid-degraded celluloses. It was found that the latter were 5 to 10 times as viscous as starch derivatives of similar molecular weight. While the viscosity of the starch derivative would be reduced if the molecule assumed

a coiled structure, this would not be compatible with its high yield of tetramethylglucose. Therefore, only a structure which was branched through glucosidic side-chains could explain all the known facts. Simultaneously, Haworth, Hirst and Isherwood[48] proposed a similar structure for glycogen, based on the observation that hydrolysis of methylated glycogen gave approximately equal amounts of tetramethyl and dimethyglucose. Freudenberg and Boppel[49] subsequently identified the latter as the 2, 3-dimethylglucose ; side chains must therefore be glycosidically attached to the main chain through the 6-position.

The interpretive studies of K. H. Meyer are primarily responsible for the present structural concepts of the starch fractions. In a series of papers, Meyer, Bernfeld and their co-workers have developed the theory that the minor component (amylose) is an unbranched linear chain of some 300 glucose units, while the major component (amylopectin) represents a ramified or branched-chain structure of several thousand glucose units.

The fractionation methods employed by Meyer are a refinement of those of Gatin-Gruzewska, leaching the more soluble amylose from the swollen starch granules at temperatures slightly above the gelatinisation point. According to Meyer, Brentano and Bernfeld,[50] a 2 per cent. aqueous suspension of corn starch was heated for one hour at 80° C. with gentle agitation, then centrifuged to precipitate the swollen granules. The supernatant solution of amylose was decanted and the precipitate re-extracted in similar manner with fresh water. After ten such digestions, the total amount of extracted solubles reached a limiting value corresponding to 35 per cent. of the original starch. At 70° C., solution of the amylose was much slower, and the yield of soluble amylose from corn starch reached 33 per cent. only after repeated extraction over a period of fifteen days. Fractionations were likewise conducted with 33 per cent. aqueous chloral hydrate at 20° C., this medium swelling the starch granule at room temperature. Repeated extractions gave a total of 27 per cent. soluble amylose.

The present writer has studied the efficiency of aqueous leaching methods as a means of starch fractionation, estimating the purity of the soluble amylose from its iodine binding capacity.[51] A 2·5 per cent. paste of defatted corn starch was extracted four successive times at the specified temperature and the supernatant solubles were recovered by alcohol precipitation. In the following table are given the amounts of total solubles (expressed as percentage of the original starch), the approximate purity of this soluble amylose (calculated in terms of pure linear-chain component), and the fractionation efficiency (representing the per-

centage of total linear-chain component recovered in the soluble amylose):

TABLE VII

Extraction Temperature	% *Solubles*	% *Purity of Solubles*	% *Fractionation Efficiency*
70°	14·3	75	39
75°	18·3	76	50
80°	20·9	73	55
85°	25·8	63	58
90°	27·1	65	63

It will be noted that aqueous leaching methods have two principal disadvantages. First, the extracted amylose is badly contaminated by concurrent leaching of branched-chain amylopectin, especially at elevated temperatures. Secondly, approximately half of the total linear-chain component retrogrades *in situ* within the swollen granule and is consequently included with the amylopectin.

As a means of purifying the crude amylose, Meyer, Bernfeld and Wolff[52] utilised its characteristic property of retrogradation. For example, 250 gm. of corn starch extracted once at 70° C. with 12·5 litres of water gave 10·4 litres of clear supernate containing 15 gm. of crude amylose. This solution was allowed to retrograde for six weeks at 5° C., giving 10 gm. of precipitated amylose. The yield and quality of the product were found to vary markedly with different samples of corn starch. Also, slight variations in the temperature of leaching produced substantial differences in the resulting amylose. These differences were attributed to heterogeneity of the amylose component, which was believed to contain linear molecules with a range of molecular weight from 10,000 to 60,000. Since the shorter chains were more soluble and more stable in solution, these were leached most readily from the swollen starch granule and were likewise the last to retrograde from an amylose solution. This diversity in molecular chain lengths was established, at least qualitatively, by intrinsic viscosities and by osmotic pressure measurements on the acetylated subfractions. By fractional leaching of corn starch at progressively higher temperatures (70°, 80° and 90° C.), Meyer, Brentano and Bernfeld[50] showed that the initially extracted amylose had a lower intrinsic viscosity and a smaller molecular weight (osmometrically) than the components subsequently solubilised at higher temperatures. Similarly, when retrograded amylose was fractionally extracted with water at 90° C., Meyer, Bernfeld and Wolff[52] found the more soluble portions to be of lower molecular weight than the more resistant portions.

By methylation in sodium hydroxide solution, followed by hydrolysis and fractional distillation, the yields of tetramethylglucose from raw corn starch, amylose and amylopectin were 3·5 per cent., 0·32 per cent. and 3·7 per cent., respectively.[53] Amylose therefore appeared to have a linear structure, with one non-aldehydic end-group per 300 glucose units, in agreement with its osmotic molecular weight of 50,000. Amylopectin had one such end-group for each 27 glucose residues; the molecule of 200,000 molecular weight must therefore be branched more than fifty times. By nephelometric methods, Meyer, Wertheim and Bernfeld[53] measured the reducing power of amylose toward ammoniacal silver oxide. While the results were at best only an approximation, they indicated that the content of aldehydic terminal groups in amylose was of the same magnitude as the content of non-aldehydic terminals; this was further evidence of the linearity of amylose.

It should be stressed that methylation analysis of amylose is enormously disturbed by the presence of relatively small amounts of amylopectin. This can be demonstrated from Meyer's yields of tetramethylglucose, assuming that his amylose contained as little as 5 per cent. of amylopectin. Correcting for the latter, the yield of tetramethylglucose from hypothetically pure amylose would be of the order of 0·14 per cent., and the amylose molecule would therefore be more than twice the length proposed by Meyer. In the opinion of the present writer, unless the amylose is of the highest possible purity (i.e. better than 98 per cent.), estimates of chain length and structure from methylation data are virtually impossible. Evaluation of average branch length of the amylopectin is probably accurate, since any amylose would be at worst merely an inert contaminant.

Meyer[54] considered amylopectin to be a highly ramified or reticulated structure, randomly branched through α-1, 6 linkages. The molecule might therefore be likened to a maple tree. He rejected the more uniform 'comb pattern' proposed by Staudinger and Husemann, which visualised a principal chain with lateral side chains of about 20 glucose units each. Meyer's argument against the comb molecule was based on observations of the action of α-glucosidase (from brewers' yeast) on the limit dextrin remaining from β-amylase conversion of amylopectin. Present theory visualises the action of β-amylase as initiating at the non-aldehydic terminals of the branched molecule, successively splitting off maltose units until a point of branching (through α-1, 6 linkage) is encountered. Thus the branched chain would be fused down to a 'stub-end' of a single glucose residue. If this

β-amylase limit dextrin were derived from a comb-type amylopectin, treatment with α-glucosidase should (according to Meyer) split off these stub-ends to give a perfectly linear main 'stem'. The latter should then give a blue colour with iodine and should be converted to 100 per cent. maltose by β-amylase. However, Meyer observed that when the β-amylase limit dextrin (Dextrin I) from amylopectin was treated with α-glucosidase, the resulting product (Dextrin II) gave a red-brown to violet colour with iodine. Moreover, when Dextrin II was again treated with β-amylase, it suffered only 18 per cent. conversion to maltose; the unattacked residue (Dextrin III) could be recovered by alcohol precipitation and gave a red-brown iodine colour. Meyer therefore visualised amylopectin as a ramified and multiple-branched molecule. If the β-amylase limit dextrin from such a molecule were treated with α-glucosidase, the resulting product (Dextrin II) would still be of highly branched character and would be subject to β-amylase action only up to another point of branching.

Meyer's argument for ramified structure depends largely on the authenticity of the α-glucosidase preparation and the correct interpretation of the action of this enzyme. However, the concept of random and multiple branching is certainly attractive. Recent work[55] in the writer's laboratory indicates that the β-amylase limit dextrin from waxy maize starch (an amylopectin type) possesses an intrinsic viscosity in 1 N KOH identical with that of the parent waxy maize starch itself. Since intrinsic viscosity is essentially a measure of relative axial ratios, this observation is in accord with Meyer's concept. If it is assumed that the waxy maize molecule is of globular character, then the action of β-amylase would merely give a smaller globular molecule, possessing the same axial ratio and hence the same intrinsic viscosity. Were the molecule of the comb-type postulated by Staudinger, then β-amylase action would markedly decrease the width of the molecule without substantially diminishing its length. The intrinsic viscosity should therefore be increased. A similar effect might be anticipated in the case of a 'laminated' or singly-branched molecule.

According to Meyer's description, amylose is inherently insoluble in water; for example, the solubilities of rice, potato and corn amyloses are given as 0·11 per cent., 0·054 per cent. and 0·04 per cent., respectively. The solubility of so-called 'natural amylose' (freshly leached from the swollen starch granule) is substantially higher, but this product is highly unstable and undergoes rapid retrogradation. The retrograded product can be restored to soluble condition by dissolving in chloral hydrate

at 80° C., precipitating with acetone and extracting with ether. The ether-moist material is soluble in boiling water to the extent of 2-3 per cent. However, it cannot be stored even in the moist state without impairment of solubility, and attempts to dry the material result in its complete insolubilisation. In the opinion of the present writer, the physical instability of amylose makes it impossible to evaluate solubility in any such exact percentage terms. By methods to be described at a later point (involving precipitation with polar reagents and special drying techniques), the linear component of corn starch has been obtained as a dry free-flowing powder. This product is completely soluble in boiling water, even at concentrations as high as 10-15 per cent. Consequently, this writer prefers to consider amylose as highly water-soluble, but subject to rapid physical aggregation to an insoluble condition. Meyer's amylose preparations were of two types, either the freshly leached solution or the retrograded product in various stages of aggregation.

Meyer[56] further differentiated the amylose and amylopectin components in the following terms:

1. Amylose is essentially phosphorus-free, while the amylopectin component (especially as derived from the tuber and root starches) contains substantial amounts of organic phosphate.

2. Retrograded amylose gives a strong X-ray diffraction pattern, corresponding to the B-spectrum described by Katz.[57] Dried amylopectin is at first amorphous to X-rays but develops crystallinity after several days' standing.

3. The ethers and esters of amylose yield strong extensible films, while the corresponding products from amylopectin are weak and brittle.[58] This is in agreement with the proposed linear structure of amylose.

4. The specific and intrinsic viscosities of solutions of amylose and its derivatives are generally lower than the corresponding values for amylopectin.

According to Meyer and Bernfeld,[59] it is the amylopectin which is primarily responsible for granule organisation and crystallinity and for the peculiar property of swelling. The starch granule is visualised as comprised of concentric onion-like layers. In each layer, the highly ramified amylopectin molecules are folded and interwoven into a reticulated network, such that the linear portions of the chains are generally oriented radially with respect to the granule. Under these circumstances, secondary valence forces (hydrogen bonding) operate tangentially to impart mechanical coherence to the granule. Certain areas in this network may be highly organised into micellar structures; other areas may be

relatively random, loosely woven and amorphous. It should perhaps be pointed out that the term 'micelle' refers to submicroscopic regions of parallel-wise orientation, and not (in the older sense) to a group of associated molecules. Thus, there may be several micellae in a single amylopectin molecule, where the branching chains are organised in side-by-side fashion. Or the 'fringes' of several adjacent molecules may be associated to give a micelle. The starch granule swells in hot water because the tangential secondary valence bonding is weakened by hydration. However, the reticulated network survives to some extent even up to 120° C., explaining the persistence of the swollen but intact granule.

This theory of granule organisation explains the preferential leaching of amylose from the swollen granule by virtue of its lower molecular weight and consequent higher diffusibility. Being of simple linear structure, the amylose would be less entangled in the complicated micellar network. This theory may also explain some of the early attempts by Naegeli and his contemporaries to fractionate starch by treating the unswollen granule with acid or with saliva. These agents might be expected to attack and solubilise the more amorphous intermicellar regions of the granule, leaving the micelle substantially intact. The granule would retain its superficial appearance but would dissolve completely in hot water without the formation of a paste, since the intermicellar network had been destroyed. It appears that Naegeli's amylodextrin represents merely the micellar residues and is not derived specifically from either of the fractions. In unpublished work in the writer's laboratory, Dexter French has obtained a product corresponding to amylodextrin by treating granular waxy maize starch (containing no amylose) with hot alcoholic hydrochloric acid; subsequent aqueous leaching removed the more amorphous portions of the granule which had been solubilised by acid hydrolysis.

Fractionation by Selective Precipitation of the Linear Component.—During the past decade, a completely different technique of fractionation has been developed by the present writer, and extended and expanded by other interested investigators. This method is based on selective precipitation of the linear starch component through the formation of insoluble complexes with various polar organic substances. Like so many chemical 'discoveries', this mode of fractionation is new in practice but old in principle. A number of earlier investigators obtained crystalline formations by treating starch solutions with polar organic reagents, but they either did not recognise that a

fractionation had thereby been obtained or else did not refine their methods to a sufficient extent. The long delay in developing this mode of fractionation must be attributed to the fact that, until recently, no adequate means has been available to differentiate, characterise and analyse the various fractions.

As early as 1926, Alsberg noted the formation of crystals when starch solutions were treated with alcohol. His associate, van de Sande-Bakhuyzen,[60] reported that when solutions of ball-milled wheat starch were treated with alcohol, rosettes of slender needles developed on standing, the individual needles being about 1 micron thick and 25 microns long. Alsberg and Griffing[61] obtained similar results with autoclaved solutions of potato, wheat, corn, canna and arrowroot starches. The spherocrystalline rosettes obtained from potato and canna starches were particularly large and well-formed, in some instances having a diameter over half that of the natural starch granule. Under crossed nicols, these gave a clearly defined ' maltese cross ' pattern.

By the action of *B. marginatum* on starch media, Lucia McCulloch[62] obtained an abundance of well-formed spherocrystals, 15-140 microns in diameter, birefringent under polarised light and blue-staining toward iodine. From the description of these crystals, it seems highly probably that they represent a complex of the linear starch component. Reschke and Hartmann[63] treated exhaustively dried potato starch with 20-30 per cent. aqueous pyridine. A microcrystalline floc formed on standing, which they considered to be an amylose-pyridine complex. In support of this belief, amylose prepared by freezing, thawing and leaching of starch pastes gave a 100 per cent. yield of the crystalline pyridine complex. The product could be dissolved in hot 30 per cent. pyridine and crystallised from this medium on cooling. Bungenberg de Jong[64] pasted starch in 30 per cent. aqueous alcohol at 70° C. On stirring, this mixture gave a ' streamer ' effect, due to polarisation of minute hexagonal platelets in the direction of flow. These crystals could be isolated by centrifuging and represented 18-19 per cent. of the starch. De Jong attributed the phenomenon to ' coacervation ' of the starch. The latter term was used in the sense employed by Kruyt, Koets and von Pryzlecki, to characterise the separation of a colloid in the form of liquid droplets ; this was presumably followed by an oriented crystallisation. Thus the fact was overlooked that an actual fractionation had been obtained.

Selective precipitation of the amylose component by certain alcohols was reported in 1941 by the present writer[65] and independently in the same year by Wiegel.[66] According to the latter

investigator, a 1 per cent. potato starch paste in dilute (30 per cent.) ethyl alcohol was refluxed for 4-5 hours, then filtered hot. On cooling, amylose separated as thin platelets, about 4 microns in diameter and 1 micron thick. These were isolated by centrifuging, washed with absolute alcohol followed by ether, and finally dried. The resulting amylose was a fluffy white powder, soluble in hot 30 per cent. aqueous ethyl alcohol, from which it recrystallised on cooling. Yield from potato starch was about 10 per cent. Wiegel found that this product gave an X-ray spectrum identical with the V-type ('Verkleisterung' or pasting pattern) described by Katz.[57] However, if the amylose was allowed to retrograde spontaneously from an aqueous solution in the absence of alcohol, it gave a B-spectrum similar to that of the native starch. According to Katz, the V-spectrum represented a special modification of the starch in freshly prepared pastes and in freshly baked bread. In contrast, Wiegel claimed that the V-spectrum was specific for amylose crystallised in the presence of alcohol, and he aptly pointed out that Katz had used alcohol for precipitation or dehydration of the starch in virtually all instances where a V-spectrum was reported. In a subsequent paper, Wiegel[67] reported that amylose can be precipitated as needles or as crystalline rosettes by such agents as isopropyl, isoamyl or butyl alcohol, dioxane or ethylene dichloride. Using a first-order red plate, these crystalline forms were found to be doubly refractive under polarised light.

According to K. H. Meyer and Maria Fuld,[68] Wiegel's amylose appeared to be a mixture of equal parts of amylose and amylopectin, since its conversion with β-amylase gave 20 per cent. of unattacked limit dextrin, corresponding to 40-50 per cent. content of amylopectin. Also, Wiegel's amylose had a higher viscosity in hydrazine hydrate solution than the amyloses which Meyer had obtained by aqueous leaching of corn and potato starches. This difference in viscosity can now be explained, since aqueous leaching extracts only the shorter amylose molecules, the longer chains retrograding *in situ* within the swollen granule. Wiegel undoubtedly obtained a partial fractionation by selective precipitation, though his recovery of amylose was incomplete and his residual amylopectin was impure.

The present writer originally observed the selective precipitation of a minor component (constituting 21-23 per cent. of corn starch) when an autoclaved solution of the starch was allowed to cool in contact with excess *n*-butyl or *n*-amyl alcohol.[65] This precipitation took the form of minute needles or spheroids, and the continuous supercentrifuge provided the best means for

isolating the microcrystalline product. The precipitated fraction was thus obtained as a creamy white paste, readily soluble in boiling water to give clear low-viscosity solutions at concentrations as high as 15 per cent. Such solutions were extremely unstable, tending to give insoluble surface 'skins' and setting to hard irreversible gels when allowed to cool. The product could be recrystallised by dissolving in boiling water and cooling in contact with *n*-butyl alcohol. In the original publication, it was erroneously stated that the precipitated fraction reverted to insoluble state when dehydrated with alcohol; subsequent studies have provided means for dehydrating the product without impairing its water solubility. The major component of the starch, not precipitated by butyl or amyl alcohol was recovered by gross flocculation of the centrifugate with methanol. This material gave viscous but stable solutions in water. In this earlier publication, alkali lability[69] provided the only chemical differentiation between the fractions, the alkali numbers of the butyl alcohol precipitated fraction and the non-precipitated fraction from corn starch being 20-25 and 5-6, respectively.

Some digression seems in order at this point to consider the nomenclature of the starch fractions. The terms amylose and amylopectin, as well as α- and β-amylose, have become hopelessly confused by application to a great variety of questionable products. On several occasions, the names of the fractions have been employed in an opposite sense; for example, Mabel Baldwin's usage of α- and β-amylose reversed the terminology of Taylor and of Alsberg. In most cases, the properties ascribed in the literature to any particular fraction were not actually those of the pure undegraded material, but were conditioned to a considerable degree by impurities, chemical degradation and retrogradation. Hence, in the interests of accuracy, it is advisable to refer to a particular product as Maquenne's amylopectin, Samec's erythro-amylose, etc. K. H. Meyer has assigned the terms amylose and amylopectin to the linear and branched molecules, respectively. However, Meyer states, 'In this to be sure, we depart from common usage, for Maquenne, who introduced these terms, used them with entirely different meanings.'[56] It is perhaps unfortunate that Meyer did not coin new names for the linear and branched components, rather than using terms which had already been corrupted by application to a wide variety of products. Furthermore, the stem '——pectin' should be reserved for those carbohydrates containing uronic acid residues. From a practical standpoint, Meyer's amylopectin includes approximately half the total amylose of the starch, and its reported

insolubility is due in large part to the retrogradation of this contained amylose. Correspondingly, his amylose represents the short-chain subfraction of the total amylose.

It has become increasingly difficult to draw an unequivocal line of demarcation between the amylose and the amylopectin. A portion of the amylose may be slightly branched, and some of the amylopectin molecules may contain long linear outer branches. There are also indications of an intermediate material representing a graded transition between the two main fractions. Until these questions are more fully clarified, it seems preferable to use purely arbitrary designations for the unique products obtained by separation with polar reagents. In the writer's laboratory, it has been found convenient to designate as the 'A-fraction' that portion of the starch which is selectively precipitated in microcrystalline state by such agents as the higher alcohols and fatty acids, and which also possess a strong affinity for iodine.[51] Unless otherwise stated, it is presumed that the A-fraction is in a soluble non-retrograded condition. Correspondingly, the 'B-fraction' is defined as the non-precipitated portion of the starch, possessing only weak affinity for iodine. These designations apply only to experimentally prepared materials and carry no connotations of purity, homogeneity or structure. However, it is a reasonable presumption that the A-fraction is relatively linear and the B-fraction relatively branched in structure. A comparison with the fractions obtained by other investigators is shown in the following table:

TABLE VIII

Investigator	*Mode of Separation*	*Linear Fraction*	*Branched Fraction*
Pacsu	Selective adsorption on cellulose	β-Amylose	α-Amylose
Samec	Electrophoresis (potato starch)	Amyloamylose Amylose	Erythroamylose Amylopectin
Taylor Alsberg	Ultrafiltration or electrophoresis (corn starch)	α-Amylose Amylopectin	β-Amylose Amylose
Baldwin	Leaching	β-Amylose Amylose	α-Amylose Amylopectin
K. H. Meyer	Leaching		

The technique of fractionation by selective precipitation of the linear component has undergone a long series of modifications and improvements in the writer's laboratory. By the original method,[65] an excess of *n*-butyl alcohol was added to a hot autoclaved starch solution and the mixture cooled to room temperature to precipitate the A-fraction. In subsequent work,[70] the starch was pasted in hot water in the presence of excess *n*-butyl alcohol, and granule structure was disintegrated by boiling under reflux for 4-5 hours or by autoclaving for several hours at 18-20

b. steam pressure. The A-fraction precipitated when the mixture was slowly cooled to room temperature. In the case of corn starch, the product crystallised as minute six-lobed spherules, 15-50 microns in diameter. The A-fraction of potato starch crystallised either as clumps of hair-like needles or more usually as beautifully formed six-petalled rosettes, 50-80 microns in diameter. These crystals were moderately birefringent under polarised light and markedly dichroic when lightly stained with iodine.

It was realised that a better separation of the starch components might perhaps be accomplished with other reagents, but no means was available at that time to assay the purity of the starch fractions. The subsequent development of potentiometric iodine titration[71] for the estimation of linear starch substance made it possible to determine the efficiency of fractionation with other reagents. The linear A-fraction was precipitated by virtually any monohydroxy aliphatic alcohol, from methanol to lauryl alcohol.[51] A number of aromatic and heterocyclic hydroxy compounds likewise proved effective, including α-terpineol, menthol, *dl*-borneol, thymol, and benzyl alcohol. In choosing the optimum reagent for the primary separation, the main requirement was that it should give a branched B-fraction of maximum purity as judged by iodine titration. While the A-fraction could be readily purified by recrystallisation, there was no acceptable method for purifying the B-fraction. From this standpoint, *n*-butyl alcohol was not as effective as other precipitants and its use is no longer recommended for the primary separation. The preferred reagents included *n*-amyl alcohol and 'Pentasol', the latter being a commercial mixture of primary amyl alcohols marketed by Sharples Chemicals, Inc. While some degree of fractionation was effected with methanol and with ethyl alcohol, the separation was distinctly inferior. Most of the higher aliphatic alcohols gave better separation than *n*-butyl alcohol.

K. H. Meyer[56] has criticised the use of autoclaving as a means for dissolving the starch substance prior to fractionation, on the grounds that the copper number is thereby increased, indicative of glucosidic scission. This is undoubtedly true of the cereal starches whose *p*H is usually between 5-5·5. However, if the cereal starches are first defatted[36] to remove natural fatty acids which give rise to undesirable acidity, and if the *p*H of the paste is buffered within limits of 5·9-6·3 during autoclaving, there is no evidence of hydrolytic degradation of the starch substance as judged by copper number[46] or ferricyanide reducing value.[72] As an even more sensitive criterion of glucosidic scission, there is no rise in the alkali number.[69] Indeed, if the composite alkali

number of the starch fractions is calculated (i.e. percentage yield of A-fraction × alkali number of A-fraction + per cent. B-fraction × its alkali number), the estimated value is identical with the alkali number of the original parent starch. Nevertheless, there is still the possibility that autoclaving may produce some minor degree of degradation, too slight to be detected by alkali number. The intrinsic viscosity of the starch fractions (discussed at a later point) has provided positive evidence of such degradation. Despite the most careful control, it has been observed that autoclaving frequently lowers the intrinsic viscosity of the starch fractions.

The following fractionation technique[55] is therefore preferred over all previous methods, since it eliminates the questionable autoclaving operation. In addition, it permits direct fractionation of the cereal starches without the nuisance of preliminary defatting. In a 5-gallon Pyrex bottle, 300 gm. of starch (calculated on moisture-free basis) is suspended in a mixture of 15 litres of water and 1 litre of Pentasol. The system is buffered at a *p*H of 6·1-6·2 by the addition of 50 ml. of 20 per cent. phosphate solution (16·4 per cent. with respect to anhydrous KH_2PO_4 and 3·6 per cent. to anhydrous K_2HPO_4). The bottle is fitted with a reflux condenser and an efficient mechanical stirrer and heated in a suitable boiling water bath. The starch suspension is heated to the azeotropic distillation temperature (92° C.) with vigorous agitation, gently refluxed for 2-4 hours, then cooled overnight and refrigerated for an additional 24 hours. The mixture is stirred continuously during the heating, cooling and refrigeration operations. The A-fraction complex crystallises as rounded spherules or crystal clusters (Fig. 6 : 1). Separation is accomplished by passing the mixture through a Sharples continuous super-centrifuge operating at 50,000 r.p.m., giving a dense well-packed deposit of A-fraction with a consistency similar to that of 'library paste'. The supernate is again passed through the super-centrifuge to remove any residual trace of linear material and the B-fraction is precipitated as a doughy mass by the addition of two volumes of methanol. Alternatively, the supernate may be treated with one-third its volume of methanol and the mixture refrigerated overnight.[73] The B-fraction precipitates quantitatively as a soft curd which is removed and dehydrated by agitation in the Waring 'Blendor' with successive portions of methanol.

The crude A-fraction can be repeatedly washed by suspending it in cold water previously saturated with Pentasol or *n*-butyl alcohol, without impairing its crystal structure. If treated with

FIG. 6.1. Pentasol-precipitated A-fraction from potato starch, stained with iodine. Magnification, 1,000 diameters.

(Reprinted from 'Outlines of Biochemistry', by R. A. Gortner, 3rd edition, John Wiley, 1949.

(*Facing p.* 146.

cold water alone, the crystallinity is destroyed and the A-fraction retrogrades to an insoluble state. This behaviour is in accord with the theory that the A-fraction is precipitated as a crystalline complex with the alcohol. Contact with water removes alcohol from the complex, causing the association and insolubilisation of the linear molecules. The crude A-fraction may be readily purified by reprecipitation with *n*-butyl alcohol. For reasons to be discussed at a later point, Pentasol does not give optimum results as a recrystallising agent. The moist A-fraction from the supercentrifuge rotor is added in small portions to actively boiling water with vigorous mechanical stirring, to give an approximate 1 per cent. solution. The crude A-fraction quickly liquefies and dissolves in the solvent medium without the formation of lumps and consequent danger of retrogradation. Excess *n*-butyl alcohol is added and the hot solution passed through the supercentrifuge, to remove dirt or cellular material carried through from the original starch. The centrifuged solution is reheated to 80-90° C., additional *n*-butyl alcohol added (if necessary), allowed to cool with stirring, then refrigerated and centrifuged. Maximum purity is generally attained after two such recrystallisations, as judged by the iodine-binding capacity of the product. After the final recrystallisation, the A-fraction is dehydrated by suspending in 10 volumes of *n*-butyl alcohol, stirring for several hours and filtering on a Buchner. This treatment is repeated several times and the final product dried *in vacuo* at 70° C. This method of drying avoids retrogradation, and the powdered A-fraction can be completely dissolved by slow addition to boiling water with vigorous mechanical agitation to prevent the formation of lumps.

It is difficult and perhaps impossible to estimate the precise content of linear material in the various starches, since different precipitants give somewhat different yields of A-fraction. As an outstanding example of this discrepancy, corn starch gives 21-23 per cent. yield of A-fraction by primary separation with *n*-butyl alcohol followed by recrystallisation with the same reagent.[70] If both fractionation and recrystallisation are conducted with Pentasol, the yield is 28-29 per cent.[51] It is believed that the difference represents material of an intermediate structural character, sufficiently linear to permit precipitation by Pentasol but with a degree of branching which prevents complex formation with *n*-butyl alcohol. For this reason, it is recommended that the primary fractionation be conducted with Pentasol, to avoid inclusion of any such intermediate material in the B-fraction. By recrystallising the Pentasol-precipitated product with *n*-butyl alcohol, the intermediate material may be removed from the A-

fraction. By fractionation and recrystallisation with Pentasol, the yields of A-fraction from various starches are as follows: tapioca, 17 per cent.; potato, 20-21 per cent.; wheat, 26 per cent.; sago, 28 per cent.; corn, 28-29 per cent. Discounting the presence of a small amount of blue-staining granules due to cross-pollination, the starches of waxy maize, waxy sorghum and waxy rice give no A-fraction with either Pentasol or *n*-butyl alcohol. It is therefore concluded that the waxy starches are composed entirely of branched type molecules,[74, 75, 76] a conclusion which is in accord with their low retrogradation tendency, their red-staining reaction toward iodine, and their low alkali number (viz. 3·6-4·1). Following recommendations by M. M. MacMasters, the writer has prepared pure waxy maize starch from certified waxy seed corn. The latter can be obtained from the hybrid seed dealers, hand-sorted to a content of less than one part per thousand of blue-staining kernels. The seed was steeped for 2-3 days in 0·5 per cent. sodium metabisulphite solution at 50° C., then ground in a suitable mill and the magma repeatedly screened through fine bolting cloth. The starch was sedimented six to eight times from distilled water, filtered, washed with alcohol and air-dried. When stained with iodine and examined under the microscope, the proportion of blue-staining granules was practically vanishing, certainly less than one part per thousand.

While the A-fraction from corn starch show exaggerated gelation and retrogradation tendencies, the corresponding linear fraction from potato starch is much more stable in solution. This divergence in physical behaviour probably reflects major differences in chain length, particularly since the alkali numbers of corn and potato A-fractions are 20-23 and 10-13, respectively. There is no such radical difference between the B-fractions from the various starches. The phosphorus content of potato starch (0·072 per cent.) is associated principally with the branched component, the crude A- and B-fractions analysing 0·01 per cent. and 0·079, respectively.[70] On electrophoresis of a solution of potato B-fraction, the latter migrated quantitatively without substantial loss of phosphorus. Samec's electrophoretic technique probably accomplished a reasonably good fractionation of potato starch, though this method would not be applicable to the cereal starches which contain only traces of esterified phosphate.

An outstanding difference between the two starch components is provided by their respective reactions with iodine. The linear A-fraction has a strong affinity for iodine, forming an intensely blue complex; the branched B-fraction has a much weaker iodine affinity and gives red or plum coloration. The

development of quantitative methods for evaluating the iodine-binding capacity represents one of the major advances in starch chemistry, since it has permitted comparison of various fractionation techniques and established the identity of the pure components. Some detailed discussion of these procedures therefore seems in order. Two general methods are in use, both depending on the iodine-binding capacity of the linear A-fraction. The latter component may be estimated spectrophotometrically by the intensity of the blue colour imparted by excess iodine, or the sample may be titrated electrometrically with iodine to determine the amount adsorbed. Both methods are relative, since they give the percentage of linear component only in terms of the intensity of blue colour or the iodine-binding capacity of some standard linear material. The spectrophotometric method of Simerl and Browning[77] for determination of starch size in paper has been adapted to evaluation of the starch fractions by McCready and Hassid.[78] According to this procedure, 100 mg. of the starch sample is introduced into a 100-ml. volumetric flask, wetted with 1 ml. of ethyl alcohol and 10 ml. of water, followed by 2 ml. of 10 per cent. sodium hydroxide solution. The sample is heated on the water bath until dissolved, then cooled and diluted to mark. Five ml. of this alkaline solution is transferred to a 500-ml. volumetric flask, diluted and slightly acidified, 5 ml. of 0·2 per cent. iodine solution added, and adjusted to volume. The light transmittancy of this solution is measured in a Klett-Summerson photoelectric colorimeter, using a red K66 filter which transmits principally in the wave-length range of 640-710 millimicrons. For a standard amylose preparation, McCready and Hassid leached the solubles from potato starch at 70° C., filtered this solution through diatomaceous earth, then precipitated the amylose from the filtrate by the addition of 20 per cent. of methanol. As evaluated by comparison against this standard, potato starch contained 10 per cent. amylose.

Kerr and Trubell[79] have modified the above method, by dissolving 100 mg. of the starch product in 10 ml. of 0·67 *N* sodium hydroxide at room temperature. This solution is diluted to about 1,500 ml., neutralised to *p*H 5 with hydrochloric acid, 20 ml. of standard iodine solution added (containing 5 gm. of iodine and 7·5 gm. of potassium iodide per litre) and finally adjusted to 2 litres. Light transmittancy at a wave-length of 610 millimicrons is determined with a Coleman spectrophotometer. As reference standard, Kerr used the corresponding 'crystalline amylose' prepared by leaching the soluble amylose from the appropriate starch paste and precipitating with *n*-butyl alcohol.[80]

On this basis, corn, potato and tapioca starches were found to contain 29 per cent., 25 per cent. and 20 per cent. of amylose, respectively. This procedure is probably more accurate than the earlier method of McCready and Hassid, since it avoids heating the starch sample in alkaline solution, which may lead to degradation of the A-fraction.

Bates, French and Rundle[71] have developed a potentiometric method for estimating the iodine affinity of the starch fractions. According to their procedure, the starch sample is dissolved in dilute sodium hydroxide solution, neutralised with hydriodic acid, then titrated electrometrically with standard iodine-potassium iodide solution. When the E.M.F. of this system is plotted against amount of iodine added, a typical sigmoid curve is obtained, and the amount of iodine in combination with the starch can be calculated from the inflection point. A more precise estimate of the 'end point' may be made by plotting free iodine against bound iodine, giving a hyperbolic type of curve. By extrapolating the legs of this curve to an intersection, the bound iodine may be readily calculated. As calibrated against Kerr's crystalline corn amylose,[80] these investigators estimate 17 per cent. amylose for tapioca and rice starches, 21-24 per cent. for corn, potato and wheat starches, 27 per cent. for sago starch, and 34 per cent. for the starch from Easter lily bulbs. Starches from the various waxy or glutinous cereals showed no iodine-binding capacity.

The present writer has adapted the procedure of Bates to the routine evaluation of iodine affinity.[55, 81] The starch substance must first be exhaustively extracted with ethyl alcohol to remove fatty acids which interfere with the sorption of iodine. An appropriate quantity of the starchy material (40 mg. of A-fraction, 200 mg. of B-fraction, or 100 mg. of whole starch) is dispersed in 5 ml. of 1 *N* potassium hydroxide and allowed to stand for several hours with occasional stirring until a perfectly clear solution is effected. The solution is then neutralised to methyl orange with 0·5 *N* hydrochloric acid, 10 ml. of 0·5 *N* potassium iodide solution added, and diluted to 100 ml. This is titrated potentiometrically with a standard iodine reagent, 0·05 *N* with respect to potassium chloride, 0·05 *N* to potassium iodide, and containing 0·2 mg. of iodine per ml. A bright platinum electrode is used in conjunction with a saturated calomel half-cell, and the E.M.F. measured with any null point potentiometer capable of being read to ± 1 mv. The starch solution is stirred mechanically during the titration, and the potential determined at some 8-12 points between 220-280 mv. For calibration purposes, an exactly

similar titration is accurately run in the absence of any starch material, in order to translate the mv. readings into mg. of free

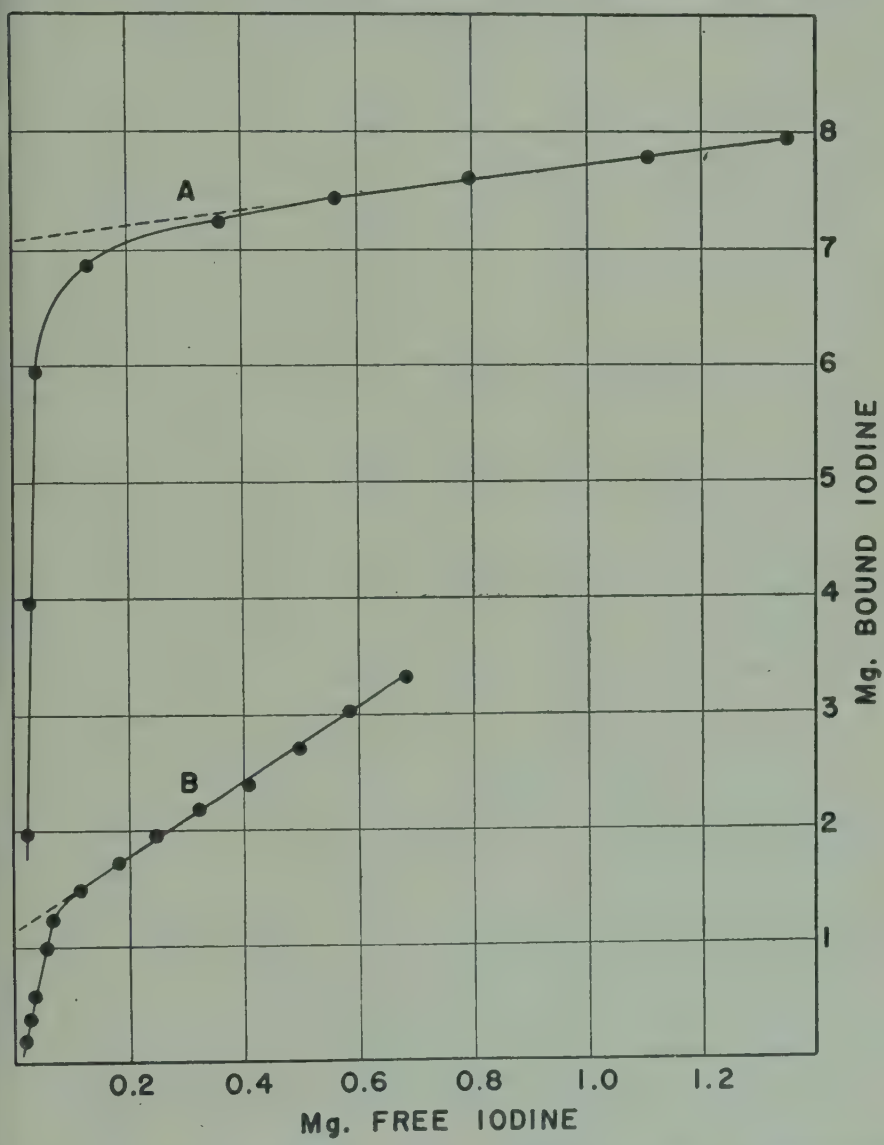

FIG. 6.2.—Iodine Titrations with Leeds and Northrup Type K Potentiometer. Curve A represents titration of 48·7 mg. of A-fraction (6·2 per cent. moisture), obtained by aqueous leaching of defatted corn starch. Extrapolation to axis gives an iodine affinity of 7·09 mg., of 15·5 per cent., on dry basis. Curve B represents titration of 197·1 mg. of corn B-fraction (7·9 per cent. moisture), as obtained by Pentasol fractionation. Extrapolation intersects axis at 1·16 mg., or 0·64 per cent. iodine affinity, on dry basis.

iodine in solution. For each point on the starch titration curve, the free iodine in solution is calculated from the corresponding E.M.F. of the calibration curve, and this value is deducted from

the total iodine added at that point to give the bound or adsorbed iodine. Free iodine is then plotted against bound iodine, as shown in Fig. 6 : 2. The upper linear portion of this curve is extrapolated back to intersect the axis, and this amount of bound iodine is taken as the iodine affinity of the starch sample.

It is preferable to express results as percentage iodine bound by the starch sample rather than calculate to an arbitrary amylose content. The main objection to the latter procedure lies in the selection of the linear type to be employed as a reference standard. For example, Kerr's crystalline amylose sorbs 20 per cent. iodine, while corn A-fraction (after repeated recrystallisations with *n*-butyl alcohol) sorbs only 19 per cent. The content of linear material can be approximated from the iodine affinities of the whole starch and the recrystallised A-fraction (e.g. 5·3 per cent. divided by 19 per cent. gives a content of 28 per cent. linear material in corn starch), but this should be regarded merely as a generalisation. Such factors as chain length and slight degrees of branching probably have a certain influence on the sorption of iodine by any particular linear substance. Typical values[55] for the iodine affinities of the fractions from various starches are included in Table IX.

Kerr and Severson[80] have superimposed butyl alcohol precipitation on fractional leaching, to obtain their so-called 'crystalline amylose'. Commercial corn starch was pasted in water at 70° C., stirred for 1 hour, then filtered. The soluble amylose leached from the swollen granules represented 5·2 per cent. of the original starch. The warm filtrate was then saturated with butyl alcohol and slowly cooled to room temperature, when the crystalline amylose separated as platelets, in an amount equivalent to 4·2 per cent. of the original starch. Similarly, potato starch gave 6·3 per cent. of soluble amylose when extracted for 30 minutes at 65° C., and subsequent precipitation with butyl alcohol gave 5·3 per cent. of crystalline amylose. The alkali numbers of these crystalline amyloses from corn and potato starches were 35 and 21, respectively, substantially higher than the respective A-fractions as precipitated by butyl alcohol. Kerr suggests that starch contains a continuous range of molecular types, from highly branched to perfectly linear, and that his crystalline amylose constitutes the more linear portion of the total A-fraction. Another possible explanation is that Kerr's crystalline amylose represents the shorter linear chains which are more readily leached from the swollen granules.[50] The low intrinsic viscosity (0·53) and high alkali number of this material would seem to confirm the latter concept.

TABLE IX

Properties of the Pentasol-separated Fractions from Autoclaved Starch Pastes; A-Fractions Recrystallised to Constant Iodine Affinity with n-Butyl Alcohol

Parent Starch	% Iodine Affinity	A-Fraction				B-Fraction		
		% Iodine Affinity	Alkali Number	Intrinsic Viscosity	D.P.*	% Iodine Affinity	Alkali Number	Intrinsic Viscosity
Tapioca (Dominican)	3·27	18·6	12·8	2·25	1300	0·0	3·8	1·23-1·27
Potato (Maine) .	4·13	19·8-20·0	12·1	1·75	930	0·4	6·4	1·45
Wheat . .	5·21	19·9	20·6	1·54	860	0·4-0·6	4·3-4·6	1·14-1·22
Corn . .	5·30	18·9-19·4	20·2-23·5	1·13-1·23	800	0·6-0·9	4·3-5·0	1·17-1·35
Sago . .	5·10	18·5	17·4	1·13	740	0·2	5·1-5·5	0·80-0·82
Easter Lily .	6·5	20·0	19·4	1·06	620	0·4	4·0	1·26

* As determined from osmotic pressure by Potter and Hassid.[82]

When a hot starch solution is slowly cooled in contact with *n*-butyl or *n*-amyl alcohol, the A-fraction separates when the temperature reaches approximately 50° C. Certain other precipitants, such as tertiary amyl alcohol or cyclohexanol, effect complete precipitation of the A-fraction at 100° C. These reagents may be added to a hot autoclaved starch solution, the mixture stirred for several minutes, then centrifuged directly. If the complex between the A-fraction and the precipitant is due to adsorption, it is conceivable that tertiary amyl alcohol or cyclohexanol would 'blanket' the linear molecule more effectively than would *n*-butyl or *n*-amyl alcohol. Similarly, cyclohexanol prevents rupture and distintegration of the starch granule. If starch is heated in water in the presence of cyclohexanol, the granules will undergo only a very restricted swelling, even at autoclave temperatures of 120° C.[73] The A-fraction is apparently insolubilised within the swollen granule by the cyclohexanol, since the filtrate from such a paste contains no substantial amount of starch substance. Cyclohexanol has little or no effect in preventing swelling and solution of waxy starch granules, a circumstance which makes it possible to remove the 3-5 per cent. of blue-staining granules which usually contaminate commercial waxy maize starch. For example, 50 gm. of the latter starch was gelatinised in a mixture of 2·5 litres of water and 250 ml. of cyclohexanol, the paste gently refluxed for several hours in a boiling water bath, then cooled and passed through the supercentrifuge. The waxy starch granules swelled and dissolved, while the blue-staining granules swelled only slightly and were deposited in the rotor of the supercentrifuge. In this manner, contaminating blue-staining granules can be removed from the branched starch substance of waxy maize.

Selective precipitation of the A-fraction can be accomplished with virtually any polar organic compound capable of hydrogen bonding. Thus, if a hot autoclaved starch solution is treated with oleic acid, the A-fraction separates as a crystalline complex with the fatty acid.[83] As with cyclohexanol, the precipitation occurs at elevated temperatures. This behaviour explains the erroneous conclusion by Taylor and his co-workers that the so-called α-amylose of the cereal starches was a fatty ester of the carbohydrate. From present information, it appears that the small amount of lipid material (0·5-0·8 per cent.) in the cereal starches is associated in the granule with the A-fraction, possibly as an adsorption complex. When the cereal starches are dispersed in water and subjected to a direct current potential, a portion of the A-fraction electromigrates by reason of the charge imparted

by this adsorbed fatty acid. Both Taylor and Alsberg considered this material to be the counterpart of Samec's amylopectin from potato starch, since both migrated in an electric field and both contained non-carbohydrate constituents. Actually, Taylor's corn α-amylose was linear in type, while Samec's potato amylopectin was branched.

Kerr's isolation of gamma-amylose from corn starch appears to be due to the formation of an insoluble complex between a portion of the A-fraction and fatty acids naturally present in the starch. When raw corn starch pastes were treated with β-amylase[84] or with the amylase from *B. macerans*,[80,85] the protective colloid effect of the B-fraction was diminished by enzyme action, and gamma-amylose flocculated in approximately 9 per cent. yield. Under similar conditions, potato starch gave no insoluble material. However, defatted corn starch gave less than 2 per cent. of gamma-amylose on conversion with *macerans* amylase, while the addition of 1 per cent. of oleic acid to potato starch resulted in the production of 18 per cent. of insoluble material.[81] The same explanation applies to the 'hemicellulose' of Schryver and Thomas and to the 'amylohemicellulose' of Ling and Nanji.

Since the A-fraction adsorbs fatty acid in preference to iodine, all starch samples should be thoroughly defatted prior to determination of iodine affinity, suitably by Soxhlet extraction for 24 hours with ethyl alcohol. The addition of about 7 per cent. of oleic acid to the A-fraction will completely repress its iodine-binding capacity; a similar effect is obtained by the addition of 2-2·5 per cent. of fatty acid to the raw starches.[83] Bates, French and Rundle preliminarily estimated 22-23 per cent. of linear component in corn starch, based on the iodine affinity of raw corn starch as compared with Kerr's crystalline amylose. Later tests on defatted corn starch have revised this value to 28 per cent., in close agreement with the yield of A-fraction by Pentasol precipitation.

Bates, French and Rundle[71] have suggested that the last traces of blue-staining material can be removed from solutions of corn B-fraction by treatment with cotton, according to the method of Tanret. However, these experiments were conducted with raw cotton, containing substantial amounts of lipid material. When high-grade surgical cotton was employed, and when this was exhaustively defatted with hot methanol, the present writer observed no decrease in the iodine affinity of corn B-fraction. Other typical adsorbents were equally ineffective, including bentonite, charcoal, activated alumina, aluminum hydroxide and kaolin. It seems probable that treatment with raw cotton merely introduced

sufficient fatty acid to repress the residual iodine affinity. In Table IX, it will be noted that only Dominican tapioca starch gave a B-fraction of zero iodine affinity. The Pentasol-separated B-fractions of the other starches all showed a small but unmistakable iodine-binding capacity. It is believed that a portion of the B-fraction may have exterior branches sufficiently long to bind a small amount of iodine, though insufficient to precipitate with Pentasol. This would be in accord with concepts recently proposed by Meyer and his associates.[86]

Bear[87] has obtained selective precipitation of the A-fraction with a variety of organic substances, including the normal and branched-chain aliphatic alcohols, the lower ketones and fatty acids, as well as various benzenoid derivatives (phenol, benzyl alcohol, aniline and benzaldehyde). The precipitates generally took the form of two- to six-lobed spherocrystals or large tufted clusters. Whistler and Hilbert[88] reported that fractionation could be effected by any reagent which possessed either donor or acceptor groups for hydrogen bonding, including alcohols, ketones, esters, carboxylic acids, mercaptans, pyridine and the nitroparaffins. Preference was given to the latter. The techniques employed for fractionation and for recrystallisation were identical with those described for butyl alcohol. However, the amount of nitroparaffin required for satisfactory fractionation was substantially less than with butyl alcohol. The yields of crude A-fraction from corn starch by precipitation with 1-nitropropane, 2-nitropropane, and nitroethane were 26-28 per cent., 29-31 per cent. and 22-23 per cent., respectively. Fractionation of potato, wheat and tapioca starches with 1-nitropropane gave 26-27 per cent., 31-32 per cent. and 20-21 per cent. of crude A-fraction, respectively.

Higginbotham and Morrison[89] have fractionated tapioca, sago and corn starches with butyl alcohol. From iodine affinity measurements, they estimate the amylose content of these three starches to be 17·5 per cent., 27 per cent. and 29·5 per cent., respectively. They ascribe the stability and low gelation tendency of the linear tapioca fraction to a slight degree of branching, rather than to variations in chain length. This viewpoint coincides with that expressed by Kerr.

Haworth, Peat and Sagrott[90] criticise butyl alcohol fractionation on the grounds that hydrolytic degradation is occasioned by autoclaving and also that butyl alcohol does not effect a perfect separation. They recommend a method whereby potato starch is pasted at 1-3 per cent. concentration, stirred for a half-hour at 100° C., 0·1 per cent. sodium chloride added, then cooled to room

temperature and saturated with thymol. On standing, the thymol complex of the linear fraction separates in 20-22 per cent. yield and is separated by centrifuging, washed and dried. Purity of the fractions is estimated by intensity of blue iodine colour, according to the method of McCready and Hassid. 'Blue values' for the potato A-fraction and B-fraction prepared by the two methods are as follows:

Precipitant	*A-Fraction*	*B-Fraction*
Thymol	0·9-1·2	0·18-0·25 (usually 0·22)
Butyl alcohol	1·05	0·18

For comparison, whole potato starch has a blue value of 0·41. Other selective precipitants include cyclohexanol, menthol and borneol. A combination of thymol and cyclohexanol gives a B-fraction with a 'blue value' as low as 0·02, though subsequent studies by Bourne and co-workers[91] indicate that this low value may be due to inadvertent hydrolysis. In comparison, the B-fractions cited in Table IX have calculated 'blue values' of 0·0-0·04, indicating that Pentasol is at least as effective as a mixture of thymol and cyclohexanol.

Higginbotham and Morrison[92] have made a careful study of iodometric methods for evaluating amylose. The potentiometric method (by a unique differential titration technique) was found to give more reliable results than the colorimetric method. These investigators likewise studied the selective precipitation of the linear fraction by various polar agents.[93] From intrinsic viscosity measurements on the triacetates of the separated fractions, it was found that autoclaving might occasion some hydrolytic degradation of the starch substance. Dispersion of the starch in the fractionating medium was therefore effected by prolonged digestion at 90° C., or by gelatinisation followed by passage through a homogeniser. However, the latter treatment gave some evidence of molecular degradation. Maximum iodine affinity of the recrystallised linear material was only achieved if the recrystallisation was conducted at very low concentrations, not exceeding 0·2 per cent. Higginbotham likewise confirms the previous observations by the present writer[51] that the residual iodine affinity of the branched component cannot be reduced by treatment with cotton. The latter merely introduces lipid material which interferes with iodine sorption.

The heterogeneity of the common starches is still not accepted by all investigators. Sutra[94] claims that the apparent linear and branched fractions are merely degradation products of the original starch substance. Similarly, Pacsu[95] visualises starch as composed of a network of glucose anhydride units, interconnected by

α-1. 4 linkages and likewise by occasional acetal bonding. Acid hydrolysis of these acetal bonds during the usual processes of gelatinisation, leaching or autoclaving presumably liberates a degradation product of linear characteristics. However, this concept does not explain the very marked differences in behaviour between the waxy and normal starches, or the fact that starches can be fractionated under alkaline conditions which could not cause scission of acetal linkages. For example, the present writer has observed a separation of the fractions when 50 mg. of corn starch is dissolved in 50 ml. of 0·2 *N* potassium hydroxide and titrated turbidimetrically with tertiary butyl alcohol, according to the method of Morey and Tamblyn.[96] When percentage light transmission is plotted against millilitres of the alcohol, there is a sharp break at 15·5 ml., followed by a second break at 27 ml. These correspond respectively to precipitation of the A- and B-fractions, as may be demonstrated by titration of the separate components. Pacsu also states that acid hydrolysis of commercial waxy maize starch produces a small amount of material which stains blue with iodine. It seems probable that this is derived from the 3-5 per cent. of blue-staining corn starch usually present in commercial waxy maize.

In summary, the majority of common starches contain a minor proportion (17-35 per cent.) of linear material and a major proportion of branched material, with perhaps a small amount of transition substance whose structure and properties are intermediate between the two main fractions. The starches of the waxy or glutinous cereals are composed entirely of branched type carbohydrate. However, Brimhall, Sprague and Sass[97] have observed that certain waxy varieties contain a small amount (2·4 per cent.) of linear substance. Waxy barley and Argentina waxy corn are of this type. These starches stain pale violet with iodine, in contrast with the deep blue of ordinary starches and the yellow brown of the more usual waxy type. Nielsen and Gleason[98] have reported that the starches from nine varieties of wrinkled peas contain 75-80 per cent. of linear blue-staining material, as judged by the colorimetric method of McCready and Hassid. On one of these varieties ('Laxton's Progress'), the present writer has obtained an iodine affinity of 15 per cent. by potentiometric titration, in close agreement with the claims of Nielsen and Gleason. The granules of these starches are compound in character. When pasted in hot water, they swell only to a very limited degree, and do not disperse even with prolonged autoclaving. For this reason, the present writer has not been able to effect a fractionation. However, Hilbert and MacMasters[99] have recently fractionated

several varieties of wrinkled pea starch (' Alderman ', ' Perfection ' and ' Stratagem ') by first dissolving the starch in liquid ammonia, according to a method credited to their associate, J. E. Hodge.[100] The starch was precipitated from ammonia medium with alcohol, then dissolved in hot water and fractionated with butyl alcohol. A yield of 60 per cent. of A-fraction was obtained, in agreement with estimates of 60-70 per cent. from iodine affinities.

Cameron[101] has observed the counterpart of wrinkled pea starch in the genetic maize variety known as ' supersugary '. A comparison with the smooth-kernel ' pseudostarchy ' type is shown in the following tabulation :

TABLE X

	Pseudostarchy	*Supersugary*
Genotype	$su^{am}su^{am}su^{am}$ DuDuDu	sususu dududu
Appearance of kernel	Opaque, smooth	Translucent, strongly wrinkled
Total sugars	Low (0·08%)	High (2·59%)
Water-soluble polysaccharides	Low (0·4%)	High (40·8%)
Granular starch in endosperm	High (80·7%)	Low (19·2%)
Appearance of starch granules	Simple	Compound
Amylose content of granular starch	Normal (31%)	High (65%)

A transition in characteristics was shown by the fourteen possible genotypes intermediate between pseudostarchy and supersugary. Cameron attributes the proportion of linear and branched fractions to genic control over the phosphorylase and isophosphorylase enzymes which are responsible for starch synthesis. By treating an aqueous solution of the water-soluble polysaccharides with two volumes of glacial acetic acid, the so-called ' glyco-amylose ', was precipitated, leaving the ' phytoglycogen ' in solution. These latter substances gave blue and brown colorations respectively with iodine and were considered as incomplete intermediates in the synthesis of the linear and branched starch fractions.

In view of the enzymatic processes responsible for starch synthesis in the plant, the starch fractions are undoubtedly heterogeneous in composition, probably comprising a graded series of molecular sizes rather than separate and sharply defined components. Speiser and Whittenberger[102] have fractionated potato starch with nitrobenzene, then subfractionated the A- and B-fractions by partial precipitation from 0·5 per cent. potassium hydroxide solution with isopropyl alcohol. Intrinsic viscosities of the products indicate a wide variation in molecular size. Kerr[103] has reported the possible subfractionation of corn A-fraction, by dissolving the latter in ethylenediamine and adding sufficient ethyl ether to give a two-phase system. That portion of the A-fraction which dissolved in the upper ether-rich phase was

isolated and recrystallised from water-butyl alcohol mixture from which it separated as rectangular platelets. The ether-poor phase, after several intermediate treatments, gave a subfraction which crystallised as star-like clusters of thin needles. Viscosity differences and crystal habit constituted the only means for characterising these products.

Kerr[104] has used the intrinsic viscosity in anhydrous ethylenediamine to demonstrate that both the linear and branched components of starch are heterogeneous and that they may be subfractionated by preferential leaching and precipitation techniques. The linear subfractions were obtained by the following procedure:

1. Aqueous extraction of defatted corn starch at 75° C., followed by precipitation and recrystallisation of the extract with *n*-butyl alcohol.

2. Aqueous extraction at 90° C. of the insoluble residue from (1), followed by precipitation and recrystallisation of this extract with *n*-butyl alcohol.

3. Solution of the insoluble residue from (2) in 0·67 *N* potassium hydroxide, followed by neutralisation and precipitation with *n*-butyl alcohol.

Corn B-fraction (separated with *n*-butyl alcohol) was separated into three subfractions by progressive additions of methanol and *n*-butyl alcohol. The properties of these subfractions are tabulated as follows:

TABLE XI

		Intrinsic Viscosity	*% β-Amylase Conversion*	*Alkali Number*
Linear subfraction	1	0·66	93	35
	2	1·17	—	34
	3	1·16	—	23
Branched subfraction	1	1·45	60	3·2
	2	0·85	55	11·3
	3	0·53	50-52	13·7

Meyer and Rathgeb[105] have employed a somewhat similar technique for subfractionation of the linear components of corn and potato starches. The water-soluble amylose ('Amylose A_1') was extracted by leaching the swollen granules twice with water at 70° C. The insoluble residue was then dissolved by autoclaving and the residual amylose ('Amylose A_2') precipitated with cyclohexanol. Degrees of polymerisation (as measured by dinitrosalicylate reducing values) of the Amylose A_1, Amylose A_2 and amylopectin from potato starch were 72, 680 and 1,100, respectively; values for the corresponding fractions from corn starch were 80-204, 2,100 and 240, respectively. The differences between the paste properties of corn and potato starches are attributed to differences in the molecular weights of their respective

fractions. Thus a corn starch paste gives a firmer gel because of the higher molecular weight of its amylose. Potato starch granules swell to a greater degree before rupturing because of the higher molecular weight of potato amylopectin. In contrast, the present writer has observed an inverse relationship between gel strength and chain length of the linear fraction. While these results appear contradictory, it must be borne in mind that the

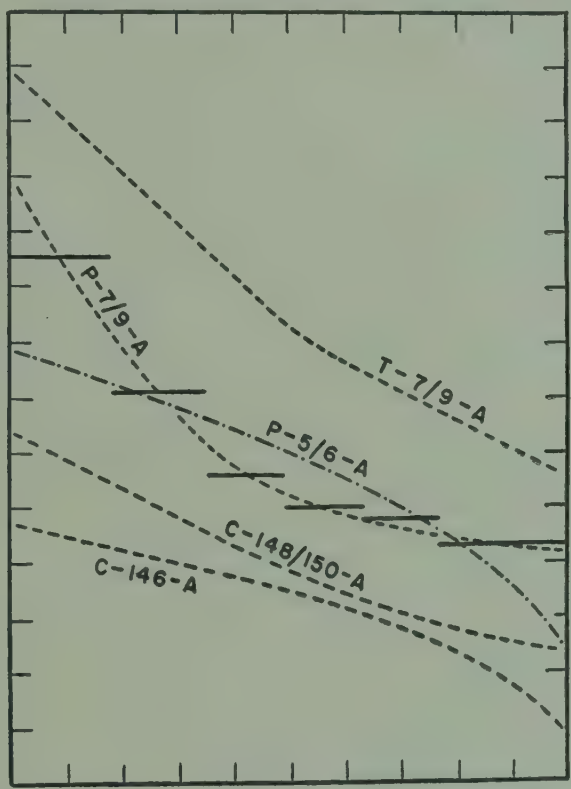

FIG. 6 : 3.—Frequency distribution of linear subfractions from various A-fractions plotted against their respective intrinsic viscosities. Method of plotting is shown for subfractions from sample P-7/9-A (Pentasol-recrystallised potato A-fraction). Other curves are as follows : P-5/6-A, butanol-recrystallised potato A-fraction ; T-7/9-A, Pentasol-recrystallised tapioca A-fraction ; C-148/150-A, Pentasol-recrystallised corn A-fraction ; C-146-A, butanol-recrystallised corn A-fraction.
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starches employed in these studies were obtained from widely different sources.

A new mode of subfractionating the linear component has recently been developed in the writer's laboratory, by successive partial precipitations with polar agents of low solubility in water.[55] It has been observed that *n*-octyl or *n*-lauryl alcohol will precipitate approximately fifteen times their weight of linear material. A preferential precipitation of the longer linear chains is effected if a

solution of the A-fraction is treated with small amounts of these agents, insufficient to precipitate the entire linear substance. In a typical instance, 100 gm. of recrystallised A-fraction was dissolved in 10 litres of boiling water, 1 ml. of *n*-octyl alcohol was added, and the mixture allowed to cool slowly to room temperature with continuous stirring. Precipitation of the crystalline complex occurred around 65° C. This was removed by centrifugation, the supernatant solution reheated to 85° C. and a second 1 ml. portion of *n*-octyl alcohol added. By successive precipitations with minimal quantities of the alcohol, the total linear material could be separated into any desired number of graded subfractions. From characterisation of the subfractions by intrinsic viscosity in 1 *N* potassium hydroxide, by alkali number and by reducing value toward dinitrosalicylate, it was apparent that the linear material of longest chain length was precipitated first, followed successively by linear subfractions of progressively shorter chain length. By plotting cumulative percentage distribution of the individual subfractions against their intrinsic viscosities, smooth curves were obtained (Fig. 6 : 3) with no indication of separate and sharply defined components. It was therefore concluded that the linear fraction was composed of a continuous ' spectrum ' of homologous linear chains. Corn and potato A-fractions from autoclaved pastes (recrystallised with *n*-butyl alcohol) gave subfractions having the following range of properties :

Source	*Intrinsic Viscosity*	*Alkali Number*	*Dinitrosalicylate Value**
Corn	0·51-1·36	21·0-28·1	4·6-9·7
Potato	0·97-2·19	9·2-19·7	2·5-4·3

Structure and Properties of the Starch Fractions.—The starch fractions offer the physical chemist an opportunity to compare the properties of two high polymeric substances of entirely different structure, yet composed of identical basic units and possessing the same total hydroxyl content. Differences in behaviour can therefore be attributed primarily to the shape characteristics of the molecules.

Hanes[106] has suggested that the starch molecule is arranged in the form of a helically-coiled spring, with 6 glucose residues per turn of the helix. Freudenberg[107] has constructed space models of such a structure, with the hydroxyl groups on the exterior of the helix. Iodine presumably enters inside the helix where the environment is essentially hydrocarbon in character ; the colour of the resulting starch-iodine complex therefore approaches that

* Reducing value expressed as mg. of maltose hydrate per gm. of starch substance.

of iodine solutions in hydrocarbon media. However, it should be pointed out that solutions of iodine in non-polar media are reddish-purple, in contrast to the pure blue starch-iodine colour.

Rundle and his co-workers have made an extensive study of the X-ray spectra and optical properties of the A-fraction complexes with iodine and with butyl alcohol. While the A-fraction appears to be stretched or extended in granular and pasted starches, they conclude that it assumes a helical form in the presence of iodine or various specific precipitants, these substances occupying the core of the helix. When starch pastes or solutions of the A-fraction were stained with iodine and subjected to high velocity gradients in flow polarisation apparatus, Rundle and Baldwin[108] observed a marked dichroism of flow. Branched types such as the B-fraction, glycogen or the waxy starches gave no evidence of alignment in the direction of flow. These observations are in agreement with the presumption of linear and globular molecules for the A- and B-fractions, respectively. Also, the long axis of the iodine molecule (I_2) must be parallel to the axis of the starch molecule. Foster and Lepow[109] studied the streaming birefringence of the linear fractions from various starches dissolved in glycerol-ethylenediamine mixtures. They observed a qualitative correlation between the intrinsic viscosity of the linear substance and its ease of orientation, since potato and tapioca amyloses polarised more readily than corn amylose. In strong solvents the amylose molecules appeared to be randomly kinked or coiled, elongating to an extended linear form in a streaming gradient. Rundle and French[110] interpreted the optical birefringence of the A-fraction crystals and their dichroism when stained with iodine on the basis of helical configuration, the axis of the iodine molecules coinciding with that of the helix. They further observed that the A-fraction in complex formation with iodine or butyl alcohol gave the V-type X-ray pattern described by Katz, while retrograded or granular starches gave A- and B-type spectra.[111] Unit cell dimensions calculated for the V-spectrum corresponded to a helical structure, 12·97 Å in diameter, with a distance of 7·91 Å between turns. This agreed with the Hanes-Freudenberg model possessing 6 glucose residues per turn. The dry A-fraction adsorbed 26 per cent. of its weight of iodine, corresponding to one iodine molecule for each turn of the helix. Rundle and Edwards[112] later obtained substantially the same unit cell dimensions for the A-fraction complex with butyl alcohol, indicating that the alcohol precipitant occupied a similar lengthwise position within the helix. While a helix possessing 6 glucose residues per turn will accommodate the linear-chain alcohols

(*n*-propyl, *n*-butyl and *n*-amyl), Bear[87] observed that the A-fraction precipitated by the branched-chain alcohols (iso, secondary and tertiary butyl) gave X-ray patterns indicative of larger unit cell dimensions. He therefore concluded that the helix enlarged to accommodate these precipitants.

From X-ray evidence, Mikus, Hixon and Rundle[113] found that the complex between amylose and fatty acid had unit cell dimensions similar to those of the complexes with iodine and with *n*-butyl alcohol. Hence they presumed that the higher fatty acids likewise induce the linear component to assume a helical structure, the fatty acid taking a lengthwise position within the helix. In agreement with observations by the present writer,[83] they found that the linear component will sorb 6-7 per cent. of its weight of oleic, palmitic or lauric acid. This value corresponded closely with the theoretical internal capacity of the helix, as calculated from space considerations. Rundle attributed the selective precipitation of the linear component by alcohols and fatty acids solely to helix formation, and not to hydrogen bonding with the hydroxyl or carboxyl group of the precipitant.

The helix theory provides an attractive and very useful explanation for the iodine and alcohol complexes. However, there are a number of anomalous reactions of the A-fraction which cannot yet be explained by present concepts of the helix. For example, the A-fraction appears to be precipitated only by polar substances (alcohols, fatty acids, nitro-compounds) and not by hydrocarbons. Yet, if the helix forms by coiling around the hydrocarbon portion of the fatty acid or alcohol, then such substances as benzene or straight-chain paraffin hydrocarbons should likewise effect a selective precipitation of the A-fraction. The hydrophilic alcohol or carboxyl group must therefore accomplish some necessary but as yet unexplained function. Secondly, it is difficult to understand why these complexes should be insoluble, since the hydroxyl loading would be identical for either the helix or the extended chain. Thirdly, the helix theory does not explain why certain hydroxyl compounds (cyclohexanol, higher fatty acids and tertiary amyl alcohol) precipitate the A-fraction at elevated temperatures (i.e. 100° C.), while precipitation with *n*-butyl and *n*-amyl alcohols does not occur until the temperature drops to approximately 50° C. In this same connection, the gelatinisation temperature of starch is lowered by the presence of the lower alcohols[114] but raised by such adjuncts as cyclohexanol or fatty acids. Fourthly, the sorption of fatty acids, iodine, or the higher alcohols by granular starch is difficult to explain on the basis of inclusion within a helix since the A-fraction in the natural starch granule is presumably

present only as extended chains. For example, granular corn starch can be 'super-fatted' with 2·25 per cent. oleic acid in methanol medium, and this added fat cannot be removed with hydrocarbon solvents.[36] This corresponds to the total sorptive capacity of the contained A-fraction. Fifthly, removal of such adsorbed materials as fatty acids or the higher alcohols from granular starches or from the A-fraction can be accomplished only by hydrophilic solvents (e.g. methanol, 80 per cent. dioxane). Hydrocarbon solvents or anhydrous dioxane are ineffective, though these should be equally capable of entering the helix. Sixthly, the blue iodine coloration of starch is completely destroyed by the introduction of less than 0·3 hydroxyethyl or carboxymethyl groups per glucose residue. It does not seem reasonable that this low degree of substitution should inhibit helix formation. Finally, as both K. H. Meyer[56] and Barger[115] have pointed out, blue iodine colorations are obtained with polyvinyl alcohol, methylcellulose, lanthanum acetate, the methylcycloacetal of acetone and a wide variety of other substances which do not appear capable of helical structure. For the present, the writer prefers to consider the selective precipitation of A-fraction on the basis of oriented adsorption of the precipitant through its hydrophilic group. Since the hydrophilic qualities of the A-fraction would thereby be reduced, its resulting insolubility might induce crystallisation. The removal of fatty acids or higher alcohols by hydrophilic solvents might be explained as due to adsorption of the solvent and desorption of the fatty acid. Admittedly, this alternative hypothesis cannot explain Rundle's excellent X-ray evidence for a helical A-fraction.

In the absence of iodine or polar agents, the physical properties of the A-fraction are essentially those of an extended linear molecule. Thus Meyer[58] has reported that the acetate of the A-fraction yields films and fibres, while the B-fraction acetate is not fibre-forming. Whistler and Schieltz[116] have obtained typical X-ray fibre diagrams for stretched films of the A-fraction acetate. Whistler and Hilbert[117] observed that films from the latter material compare in tensile strength with those from cellulose acetate. In contrast, the B-fraction acetate gave weak films which were not self-supporting.

The spontaneous deposition of insoluble A-fraction from dilute solution probably represents the formation of oriented aggregates, through side-by-side association of extended linear chains. Katz has shown that retrograded starch gives a B-type X-ray pattern, indicative of linearly associated chains. When a hot 3-10 per cent. solution of corn A-fraction is cooled to room temperature,

it sets up to a rigid gel which cannot be liquefied by heating. This type of retrogradation cannot be attributed to an orderly association throughout the gel, since the rapidity of gelation precludes such orientation. It is therefore presumed that an interlocking network is developed by local associative bonding of each linear chain with a number of other neighbouring chains. Potato and tapioca A-fractions do not retrograde from solution as readily as the linear components of corn and wheat starches, nor do they exhibit the pronounced and irreversible gelation tendencies of the latter. The greater length of tapioca A-fraction appears to impede its free orientation in solution and thus interfere with association. Indeed, within certain limits, the retrogradation tendency of linear starch substances is inversely related to chain length. Thus, Whistler and Johnson[118] have found that the rate of retrogradation of potato amylose increases progressively as the chain length is shortened by acid hydrolysis. A comparative index of chain length was provided by measurement of the specific viscosity of an 0·4 per cent. solution of the linear substance in 1 *N* potassium hydroxide. Maximum retrogradation was observed with linear substances of 0·5 viscosity; below this level, the rate of retrogradation decreased as the linear fragments became too short to associate. The present writer has observed a similarly inverse correlation between retrogradation tendency and intrinsic viscosity of the linear subfractions from various starches.[55] Subfractions of similar intrinsic viscosity exhibited substantially identical inclination to retrograde, whether derived from corn, potato or tapioca starch. Hence the theory is no longer tenable that the low retrogradation tendency of tapioca A-fraction is due to a forked or branched molecular structure.

The B-fractions from the various starches are relatively stable in solution. Under certain circumstances, insoluble aggregates may be formed but these are readily reversible by moderate heating.[73] If 10-15 per cent. of methanol is added to a solution of B-fraction and the mixture refrigerated, the B-fraction is quantitatively precipitated in the form of insoluble spherules. Similarly, concentrated solutions of the B-fraction undergo progressive insolubilisation at room temperature. These reactions are not due merely to mechanical entanglement of exterior branches, since a B-type X-ray pattern is developed, indicative of oriented association. This phenomenon is believed to be the main factor responsible for the staling of bread, a theory which is supported by the observation of Noznick and Geddes[119] that bread baked from synthetic mixtures of wheat gluten and waxy maize starch shows a normal staling reaction. It seems probable that aggregation of the B-

fraction is due to the formation of ' fringe micellae ' between the exterior branches of neighbouring branched molecules, in accord with concepts proposed by K. H. Meyer. Since these exterior branches are much shorter (e.g. 25-30 glucose units) than the chain length of the A-fraction, the insolubilised B-fraction is readily dissolved by moderate heating.

Some of the recent emulsifiers which promote softness in bread (e.g. polyoxyethylene monostearate) form complexes with the A-fraction but have no apparent affinity for the B-fraction. Hence the softer crumb consistency of the bread may perhaps be attributed to decreased gel formation by the A-fraction in the freshly baked bread, and not to prevention of aggregation of the B-fraction. The evidence for complex formation between polyoxyethylene monostearate and the A-fraction may be summarised as follows :

1. When added to a hot autoclaved solution of corn starch, the emulsifier selectively precipitated the A-fraction, though the efficiency of fractionation was poor (i.e. iodine affinities of the precipitated and non-precipitated fractions were 7·4 per cent. and 1·1, respectively).

2. Like cyclohexanol, polyoxyethylene monostearate markedly restricted swelling and prevented solution of normal starch granules on prolonged cooking, but had no such inhibitory effect on waxy granules.

3. The iodine affinity of the A-fraction was proportionately reduced by the addition of increasing amounts of polyoxyethylene monostearate. By plotting iodine affinity against added percentage of commercial emulsifier, it was calculated that the A-fraction was capable of binding 25 per cent. of its weight of emulsifier (cf., 7 per cent. in the case of oleic acid).[83]

4. Iodine titrations were run on the A-fraction in the presence of minimal amounts of polyoxyethylene monostearate exactly as described under (3) above, but with the further addition of large amounts of B-fraction. If the B-fraction had any affinity for the emulsifier, it should sequester the latter and thus raise the apparent iodine affinity of the A-fraction. However, the ' breaks ' in the titration curves were not displaced by the addition of B-fraction.

From classical methylation studies, the B-fraction was pictured as a branched type of molecule, composed of short linear branches of 25-30 α-1, 4-linked glucose units, interconnected through α-1, 6 linkages. Numerous unsuccessful attempts have been made to isolate the hypothetical disaccharide involved in branching. Using chromatographic techniques, Edna Montgomery and her co-workers[120] finally separated 6-[α-D-glucopyranosyl]-D-glucose (' brachiose ')

from the enzyme conversion residues from waxy maize starch. No such disaccharide could be obtained from the A-fraction. The concept of the fractions as linear and branched molecules is further supported by evidence from β-amylase action. Presumably, this enzyme attacks the non-aldehydic terminal of the starch molecule, progressively splitting off maltose units until the conversion is halted by a branch point. Theoretically, the A- and B-fractions should give 100 per cent. and approximately 50 per cent. yields of maltose, respectively. However, since the starch substance must be completely dissolved for β-amylase action, minor aggregation effects may lower the conversion level to an appreciable extent. Another disturbing factor is the difficulty in obtaining pure authentic β-amylase, free from α-amylase and maltase. Despite careful procedures, Whistler and Hilbert[88] were unable to obtain β-amylase conversion limits which they considered reliable. Hodge, Montgomery and Hilbert[121] obtained 53-57 per cent. maltose from the B-fractions of a number of tuber and cereal starches. To avoid association or retrogradation, Bernfeld[122] dissolved the starch fraction in alkali and added this solution to a large excess of β-amylase buffered at p_H 4·8. Under these circumstances, the A-fraction gave a 100 per cent. yield of maltose,[123] while potato and corn B-fractions gave 59 per cent. and 62 per cent. respectively.[86]

The enzyme from *B. macerans*[124] has an action somewhat analogous to β-amylase, attacking the non-aldehydic terminals of the starch molecules and splitting moieties which cyclise to the crystalline Schardinger dextrins. This enzyme may have advantages over β-amylase, since its action appears to be more specific and the Schardinger dextrins can be directly isolated as the trichloroethylene complexes. Kerr[125] has reported a yield of at least 70 per cent. of Schardinger dextrin by the action of *macerans* amylase on his crystalline amylose. Wilson, Schoch, and Hudson[81] have obtained 69-72 per cent. of Schardinger dextrin from the crude corn and potato A-fractions, with only 10-12 per cent. of residual limit dextrin. In contrast, the corresponding crude B-fractions gave 47-50 per cent. of Schardinger dextrin and 40-44 per cent. of residual limit dextrin. Theoretically, the original terminal aldehyde groups of the B-fraction should be concentrated in the limit dextrin from *macerans* conversion. Actually, the alkali number of the limit dextrin is generally lower than that of the parent material, indicating the possibility of side-reactions.

Considerable controversy has arisen over the type of branching in the B-fraction. The earliest concept pictured a 'comb' or

'herring-bone' structure, comprising a central spine to which were attached numerous short branches of 20-25 glucose units in length. This was superseded by the laminated or singly-branched structure advocated by Haworth and his associates. This may be likened to a 'feather-stitch' pattern,

a succession of short linear segments of 25-30 glucose units, each connected to the previous segment by an α-1, 6-linkage. In its earliest form, the branch points were designated by arrows, since there was some doubt whether this linkage was accomplished by primary valence or by physical aggregation through hydrogen bonding. Finally, K. H. Meyer and also Myrbäck[126] have proposed a ramified or multiple-branched pattern, a bushy structure whose gross shape is essentially globular and three-dimensional.

The choice between single and multiple branching will probably depend on a more exact elucidation of the enzymatic processes by which the fractions are synthesised in the plant organism. Although it is not within the province of this chapter to discuss starch synthesis in detail, a brief summary of the enzymatic mechanism seems appropriate. The synthesis of starch-like polysaccharides from glucose-1-phosphate has been accomplished *in vitro* by Hanes[127] and by the Coris.[128] Two enzymes are apparently required, the first to effect a linear condensation of glucose units, the second to initiate points of branching. Bourne and Peat[129] have suggested that these be designated as the P-enzyme and the Q-enzyme, respectively; alternative designations are phosphorylase and isophosphorylase. The action of P-enzyme alone would give rise to the A-fraction, while the P-enzyme in conjunction with the Q-enzyme would result in a B-fraction type. Of the various branched polysaccharides, glycogen appears to be most highly ramified, with an average branch length of only 10-12 glucose units, while the B-fractions of various starches range from 20 to 32 glucose units per branch.[130] From present information, it cannot be determined whether branch length depends on the relative concentration of the two synthesising enzymes, or whether some more complex mechanism is involved. These enzyme systems require the presence of an activating substance (usually glycogen) for the production of polysaccharide from glucose-1-phosphate. It is interesting to note that corn B-fraction prepared by Pentasol separation is effective as an activator, while recry-

stallised corn A-fraction has only a slight catalytic action.[131, 132] However, the activity of the A-fraction increases markedly with slight acid hydrolysis.[132, 133]

Thus the P-enzyme appears to build up a linear chain by attaching successive glucose units to the non-aldehydic terminals of the activator. When the linear chain is sufficiently long, the Q-enzyme may induce a point of branching, with subsequent continuation of linear synthesis on both terminals. As an alternative viewpoint,[129] the P-enzyme may build up short linear chains of approximately 20 glucose units (Peat's ' pseudo-amylose ') which the Q-enzyme then condenses to a branched structure. According to Meyer, branching may be initiated at any point within the molecule and not necessarily confined to the exterior branches.[86] In this case, the older inner portions of the branched molecule (i.e. nearest the terminal aldehyde group) would be more highly ramified than the outer branches, due to longer exposure to the action of the Q-enzyme. In any event, a clarification of the mechanisms of enzymatic synthesis should provide definite evidence of the type of branching in the B-fraction. In addition to synthetic action, there is likewise the possibility that the Q-enzyme may effect a transformation of the A-fraction into a branched type of starch substance. Thus Peat and his co-workers[134] have observed that the action of Q-enzyme on the thymol-precipitated A-fraction from potato starch gives a soluble non-retrograding polysaccharide which stains red with iodine and which has a β-amylase conversion limit of 55 per cent. Analogous to the phosphorylase system, Hehre[135] has isolated a bacterial amylase (' amylosucrase ' from *Neisseria perflavia*) which acts on sucrose to give a branched polysaccharide of starchy type.

It is difficult to understand how both linear and branched fractions can be produced simultaneously in the same starch granule in the presence of Q-enzyme. Wolf and MacMasters[136] have isolated the starch from dent corn at various stages of maturity. The iodine affinity was found to be only 1·3 per cent. at 13 days after pollination, increasing to a normal value of 5·3 per cent. at maturity. These observations would indicate that the A-fraction was formed during the later stages of ripening. In the case of the starch from growing potato tubers, Halsall and his co-workers[137] found no appreciable change in iodine affinity at various stages of growth.

Intrinsic viscosity affords a simple and useful means for characterising the physical dimensions of the starch fractions, though quantitative evaluation of molecular size from such data is not possible. For a given type of polymeric substance, the intrinsic

viscosity merely provides a measure of molecular shape, the ratio between length and diameter of the molecule. Foster and Hixon[138] have determined the intrinsic viscosities of the following A-fractions in anhydrous ethylenediamine : potato, 2·58 ; tapioca, 2·11 ; Easter lily, 1·24 ; corn, 0·91 ; Kerr's 'crystalline amylose', 0·53. If it is assumed that these substances are strictly linear, differing only in chain length, then the molecular weights are in the same relative order. On this assumption, Baldwin, Bear and Rundle[139] have calculated the chain lengths of the above A-fractions as 500, 450, 310, 250 and 175 glucose units, respectively. By comparing the absorption spectra of the iodine complexes, they find that the iodine coloration is shifted toward the red as the chain length decreases. For this reason, spectrophotometric evaluation of 'blue value' may be subject to slight errors when the iodine colorations of long and short chain molecules are compared. Marjorie Swanson[140] has determined the spectral absorption curves of the iodine complexes of various short chain fragments. Chains of 4-6 glucose units gave no iodine colour, those of 8-12 glucose units gave a red colour, while chains of more than 30 units gave a blue.

The writer prefers to use 1 *N* potassium hydroxide as a solvent for evaluation of intrinsic viscosity, rather than such heteropolar agents as ethylenediamine or chloral hydrate which may conceivably cause 'bridging' between molecules. With any basic solvent, the viscosity of the system decreases on standing, presumably due to progressive oxidative degradation of the carbohydrate. In devising a method for determining intrinsic viscosity, speed of operation would therefore seem to be a prime requisite, to dissolve the sample and evaluate its viscosity at various concentrations within several hours' time. In general, the A-fractions of the various starches (even when completely retrograded) can be readily dissolved by gentle stirring in cold 1 *N* potassium hydroxide. The B-fractions are much more resistant, apparently due to some sort of aggregation. This may be overcome by first dissolving the sample of B-fraction in boiling water, cooling to room temperature, then adding the alkali. The final concentration of potassium hydroxide must not deviate from 1 *N* by more than ± 1 per cent. The specific viscosities at 35° C. are measured at various concentrations from 0·1 to 0·5 per cent., using a Cannon-Fenske No. 100 pipette, and the intrinsic viscosity is determined in the usual fashion, by plotting the function (specific viscosity/concentration) against concentration and extrapolating to zero concentration. The intrinsic viscosities of the Pentasol-separated fractions from various starches are given in

Table IX. Intrinsic viscosity has provided a useful and sensitive index of minor hydrolytic degradation of the starch fractions. As a typical instance, prolonged disintegration in the Waring 'Blendor' is not recommended as a means for dispersing starch pastes, since the high speed shearing action appears to cause a 'mechanical hydrolysis' of the molecule. When a 3 per cent. solution of corn B-fraction was 'blended' for 1 hour, its intrinsic viscosity dropped from 1·17 to 0·95.

Beckmann and his co-workers have investigated ultracentrifugal methods for the evaluation of 'particle weights' and shape factors of the starch fractions. Sedimentation constants were determined on the acetates of Kerr's 'crystalline amyloses' from tapioca, corn and potato starches, dissolved in methyl acetate or s-tetrachloroethane.[141] The molecular weights corresponded to 650, 380 and 240 glucose units, respectively; these values are of the same order of magnitude as estimates from reducing value and methylation data. However, the 'particle weight' of the acetate of corn B-fraction in methyl acetate solution indicated a D.P. of 60,000,[142] far higher than might be anticipated from reducing value. In general, there is major disagreement between molecular weights as evaluated by physical and by chemical methods, especially with the branched component. This may be due to associative aggregation or even to molecular entanglement. From correlation of ultracentrifugal data with intrinsic viscosities and diffusion constants, Beckmann concludes that amylose acetate assumed a helical form when dissolved in methyl acetate. The calculated diameter of the helix coincides with the value suggested by Rundle and French[111] from X-ray studies.

Evaluation of molecular weight from osmotic pressure is subject to similar doubts with respect to the state of molecular dispersion, the possibility of molecular aggregation or entanglement, and the major variations observed with various solvents. For example, Cleveland and Kerr[143] have found that a sample of corn A-fraction acetate gave an osmotic molecular weight corresponding to 465 glucose units in chloroform solution, 675 in pentadione and 1,370 in pyridine. Yet each of these solvents dissolved the acetate to give a stable solution of sparkling clarity. Until osmotic molecular weights can be reconciled with values by other methods, Kerr prefers chloroform as a solvent because it gives minimum molecular weight. The linear subfractions prepared by ether-ethylenediamine phase separation of corn A-fraction showed a wide variation in osmotic molecular weight, with D.P. values ranging from 250 to 675. Kerr and Cleveland[144] observed that fully acetylated corn B-fraction did not dissolve sufficiently well in chloroform to

permit evaluation of osmotic molecular weight. On the theory that the terminal branches were causing intermolecular aggregation, they prepared the β-amylase limit dextrin of corn B-fraction, in a yield of 46 per cent. The acetate of this product dissolved readily and completely in chloroform. Osmotic measurements indicated a D.P. of 810; hence the calculated molecular weight of the parent B-fraction corresponded to a D.P. of 1,800.

Potter and Hassid[145] have determined the osmotic molecular weights of the fractions from a variety of starches. To avoid degradation, acetylations were conducted at room temperature in formamide medium. Molecular weights of the various A-fractions calculated to the following D.P. values: tapioca, 1,300; potato, 930; wheat, 860; corn, 800; sago, 740; Easter lily, 620. It is interesting to note that the intrinsic viscosities of these A-fractions fell in exactly the same order. Osmotic molecular weights of the corresponding B-fractions ranged from one to six million and showed only fair correlation with intrinsic viscosity.

The little-known Barger isopiestic method has been employed by Caesar[146] for evaluation of the molecular weights of various starch products. The starches were nitrated with nitrogen pentoxide under conditions which produced minimum hydrolytic degradation, and the molecular weights of the nitrates were determined in methyl or ethyl acetate solution. While investigations were primarily concerned with pyrodextrins, D.P. values of corn and potato amyloses were found to be 220 and 980, respectively.

Since each starch molecule (whether linear or branched) has only one aldehydic terminal, some measure of reducing value would seem to afford the best chemical measure of molecular weight. Unfortunately, all analytical oxidants for terminal aldehyde groups likewise appear to effect oxidative degradation of non-terminal glucose units. While this over-oxidation is very slow, the great preponderance of groups subject to secondary oxidation appears to vitiate any absolute evaluation of terminal aldehyde groups. The present writer has studied the rates of oxidation of corn A-fraction with a wide variety of reagents, including hypoiodite, various alkaline copper complexes, ferricyanide, bromine, and alkaline dinitrosalicylate. In all cases, the reducing value was influenced to a major degree by such factors as temperature, reaction time, *p*H level, and relative proportion of oxidant. With ferricyanide, the variation due to proportion of oxidising agent can be corrected by plotting the reducing value against time of oxidation and extrapolating to zero time. In this instance, however, no stoichiometric relationship is

observed between glucose, maltose and French's maltoheptaose; hence quantitative evaluation of aldehydic end-groups is extremely questionable. At best, it appears that reducing value gives only a proportionate measure of molecular size. However, Meyer and his associates have adapted the colorimetric dinitrosalicylate method of Sumner to the evaluation of terminal aldehyde groups.[147] The method was standardised on lichenin and on various linear starch fractions whose molecular weights had previously been established by osmotic pressure determinations. In this manner, it was possible to compensate for over-oxidation.

Frequent efforts have been made to devise a simple method of end-group analysis to replace the tedious methylation assay. The most promising method depends on the specific cleavage of glycol groups by periodate. When a linear starch chain is treated with this oxidising agent, the glucose unit at the aldehydic terminus will yield two moles of formic acid (from carbons 1 and 2) and one mole of formaldehyde (from carbon 6). The non-aldehydic terminal glucose will give only one mole of formic acid (from carbon 3), while non-terminal glucose units will merely undergo cleavage between carbons 2 and 3. Caldwell and Hixon[148] were among the first to use this reaction for the estimation of molecular weight, by determining the amount of formaldehyde produced when various dextrins were oxidised with periodic acid. They obtained molecular weights in close agreement with values by the copper reduction method of Richardson and Farrow.[46] Since molecular weights as high as 134 glucose units were obtained on some of their dextrins, they argued that the molecular weight of starch itself must be considerably higher than the currently accepted 25-glucose chain proposed by Haworth.

More recently, periodate oxidation has been employed by Hirst and his associates[130] to determine the proportion of *non*-aldehydic terminal glucoses from the amount of formic acid developed. The starch substance was oxidised with periodic acid (or preferably with potassium periodate[149]), and the formic acid titrated with barium hydroxide. Branch lengths were obtained ranging from 20 glucose units for waxy maize starch to 32 glucose units for sweet potato amylopectin; these data were in close agreement with methylation assay for tetramethylglucose. Similarly, the branch length of glycogen was estimated at 12 glucose units, while the chain length of amylose was approximately 250 units. Halsall, Hirst and their co-workers[150] have likewise used the periodate reaction to explore the possibilities of anomalous linkages in starch. If any of the non-terminal glucose units had branches attached at either carbon 2 or carbon 3, these glucose

units would not be oxidised by periodic acid. Halsall and Hirst therefore oxidised various starches with periodic acid, hydrolysed the product, and tested for glucose by chromatographic techniques. In every case, a trace of glucose was found, but in such small quantity that its significance was questionable.

Potter and Hassid[151] have further improved the periodate reaction by using sodium metaperiodate and conducting the oxidation at 2° C. They find that the branch lengths of the B-fractions from various starches range from 22 to 27 glucose units. When this technique was applied to the A-fractions from potato and Easter lily starches, end-group values were obtained which were in close agreement with the osmotic molecular weights.[145] However, tapioca A-fraction appeared to have two non-aldehydic terminals per molecule, while wheat, corn and sago A-fractions had three. Hassid therefore concluded that the molecular structures of these last four A-fractions were slightly branched.

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240. R. M. Hixon, 'Some recent developments in starch chemistry', *The Bakers Digest*, April, 1943, **17**, 19-21. (Brief semi-popular review.) *C.A.*, **37**, 3295.

241. R. M. Hixon, 'The waxy starches', *Wallerstein Lab. Comm.*, 1943, **6**, 143. (Character of waxy maize and sorghum starches. Brief abstract of paper, presented at Chemurgic Conference.) *C.A.*, **38**, 5685.

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contact with liquid surfaces such as amyl alcohol or fat, starch is adsorbed at the interface from aqueous solution. Liquid paraffin interfaces have no action. Adsorption occurs only with polar molecules (alcohols, fatty acids, esters.) *C.A.*, **27**, 1802. (Note: Compare with selective precipitation of A-fraction by alcohols and fatty acids.—T. J. S.)

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250. J. Janicki, 'Several reflections on the theory of amylopectin, in reply to M. Samec', *Roczniki Chem.*, 1933, **13**, 618-621. (Polemic.) *C.A.*, **28**, 5497.

251. J. Janicki and K. Kasprzyk, 'Compounds of serum proteins with polysaccharides', *Biochem. J.*, 1936, **30**, 1526-1531. (Phosphorus-free amylose is capable of binding proteins.) *C.A.*, **30**, 8271.

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253. J. R. Katz and T. B. v. Itallie, 'The physical chemistry of starch and bread-making. 7. The Roentgen spectra of the two constituents of starch: amylopectin and amylose', *Z. physik. Chem.*, Abt. A, 1931, **155**, 199-207. (Freshly prepared amylose and amylopectin give a V-spectrum. Both fractions retrograde, subsequently giving a B-spectrum.) *C.A.*, **26**, 2910.

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258. R. W. Kerr, O. R. Trubell and G. M. Severson, 'The multiple amylose concept of starch. 2. Amylopectin and amylose', *Cereal Chem.*, 1942, **19**, 64-81. (Gamma-amylose is not identical with amylopectin. No fractions have been obtained by any method which correspond to Maquenne's definitions of amylose and amylopectin.) *C.A.*, **36**, 2175.

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260. M. Kholodova and I. Markevich, 'Flocculation of starch by saliva', *Colloid J.* (U.S.S.R.), 1938, **4**, 615-620. (Saliva protein cause precipitation of amylopectin but not amylose.) *C.A.*, **33**, 6354.

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amylase, beta-starch by alpha-amylase. Glycogen consists almost entirely of alpha-starch.) *C.A.*, **27**, 109.

262. P. Koets, 'Complex coacervation of amylophosphoric acid and proteins and its possible bearing on the problem of amylopectin', *Proc. Acad. Sci. Amsterdam*, 1935, **38**, 63-74. (Amylose does not form coacervates with protein. Introduction of phosphate ester groups into amylose gives a product which exhibits coacervation.) *C.A.*, **29**, 2985.

263. P. Koets, 'Coacervation of amylophosphoric acid and proteins', *J. Phys. Chem.*, 1936, **40**, 1191-1200. (The mixing of solutions of amylophosphoric acid and protein causes precipitation of microscopic droplets.) *C.A.*, **31**, 2494.

264. D. Kreger, 'Configuration and packing of chain molecules of native starch in the β-modification', *Nature*, 1947, **160**, 369. (Calculation of unit cell dimensions from X-ray data.) *C.A.*, **42**, 781.

265. K. G. Krishnaswamy and A. Sreenivasan, 'Separation and determination of the amylose and amylopectin fractions of starch', *J. Biol. Chem.*, 1948, **176**, 1253-1261. (Comparative study of thymol and butyl alcohol as fractionating agents.) *C.A.*, **43**, 2455.

266. D. Krüger and E. Tschirch, 'The iodine—starch problem. 2. The colored iodine compounds of basic salts of the rare earths', *Ber.*, 1930, **63B**, 826-836. (Certain lanthanum salts give a blue iodine colour similar to that of starch. The reaction is discussed from the standpoint of particle size and adsorption.) *C.A.*, **24**, 5656.

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269. O. Lamm, 'Measurements of concentration gradients in sedimentation and diffusion by refraction methods. Solubility properties of potato starch', *Nova Acta Regiae Soc. Sci. Upsaliensis*, 1937, **10**, No. 6, 115 pp. (Diffusion and ultracentrifuge measurements indicate that potato starch solutions are polydisperse.) *C.A.*, **31**, 7722.

270. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch. 1. The process of grinding', *J. Soc. Chem. Ind.*, 1941, **60**, 1-6. (Wheat starch is ground in a ball-mill for various periods of time up to 4,000 hours, and the products separated into a cold-water-soluble fraction (I), a hot-water-soluble fraction (II), and a hot-water-insoluble fraction (III). Effect of dry-grinding is to increase progressively the more soluble fractions.) *C.A.*, **35**, 8340.

271. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch. 2. The reducing powers of the starch fractions', *J. Soc. Chem. Ind.*, 1941, **60**, 25-29. (Ferricyanide reducing values of the various fractions from ball-milled starch increase with progressive grinding.) *C.A.*, **35**, 8341.

272. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch. 3. The viscosity of starch solutions', *J. Soc. Chem. Ind.*, 1941, **60**, 47-50. (The viscosity of the hot-water-soluble fraction (II) from ball-milled starch decreases markedly with progressive grinding. Viscosity of the cold-water-soluble fraction (I) remains practically constant.) *C.A.*, **35**, 8341.

273. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch. 4. X-ray diffraction patterns of starch fractions', *J. Soc. Chem. Ind.*, 1941, **60**, 69-72. (The X-ray pattern of wheat starch is

destroyed by ball-milling. Spectra of the fractions (I, II, and III) differ from that of the ball-milled starch.) *C.A.*, **35**, 8342.

274. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch. 5. The absorption spectra of the starch-iodine complex', *J. Soc. Chem. Ind.*, 1941, **60**, 99-111. (Ball-milling causes a decrease in absorption of the longer wave-lengths by the iodine-starch complex. This is interpreted to indicate that the primary action of ball-milling is scission of the lateral linkages of the starch (i.e. rupture at the points of branching).) *C.A.*, **35**, 8342.

275. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch. 6. The precipitation of starch from aqueous solution by alcohol', *J. Soc. Chem. Ind.*, 1941, **60**, 137-141. (Both the cold-water-soluble and hot-water-soluble fractions (I and II) from ball-milled starch are insolubilised by evaporation of aqueous solutions to dryness. By precipitating and dehydrating with alcohol, only fraction II is insolubilised.) *C.A.*, **35**, 8343.

276. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch. 7. Retrogradation of starch solutions', *J. Soc. Chem. Ind.*, 1941, **60**, 175-184. (The hot-water-soluble fraction (II) from ball-milled starch shows greater tendency to retrograde than the cold-water-soluble fraction (I).) *C.A.*, **35**, 8343.

277. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch 8. Phosphorus of wheat starch', *J. Soc. Chem. Ind.*, 1941, **60**, 231-241. (The hot-water-soluble fraction (II) from ball-milled wheat starch contains 60-80 per cent. of the total phosphorus of the starch.) *C.A.*, **36**, 7341.

278. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of wheat starch. 9. Summary and discussion', *J. Soc. Chem. Ind.*, 1941, **60**, 301-305. (Summary of References 270-277. The hot-water-insoluble fraction (III) corresponds to amylopectin, the hot-water-soluble fraction (II) to amylose, and the cold-water-soluble fraction (I) to soluble starch.) *C.A.*, **36**, 7341.

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280. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of potato starch. 1. The process of grinding', *J. Soc. Chem. Ind.*, 1947, **66**, 417-420. (Potato starch is more readily solubilised by ball-milling than is wheat starch.) *C.A.*, **42**, 3982.

281. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of potato starch. 2. The reducing powers, mean molecular weights, and solubilities of the fractions', *J. Soc. Chem. Ind.*, 1948, **67**, 38-40. (Reducing values indicate that ball-milling degrades potato starch more rapidly than wheat starch.) *C.A.*, **42**, 4776.

282. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of potato starch. 3. The viscosities of the aqueous solutions of the fractions', *J. Soc. Chem. Ind.*, 1948, **67**, 41-44. (The cold-water-soluble and hot-water-soluble fractions from ball-milled potato starch have higher viscosities than the corresponding fractions from ball-milled wheat starch.) *C.A.*, **42**, 6561.

283. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of potato starch. 4. The absorption spectra and colour intensities of the starch-iodine complexes', *J. Soc. Chem. Ind.*, 1948, **67**, 97-101. (Contrary to previous observations with wheat starch, the cold-water-soluble fractions from ball-milled potato starch contain more amylose than the hot-water-soluble fractions.) *C.A.*, **42**, 7074.

284. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, 'The fractionation of potato starch. 5. The phosphorus of potato starch', *J. Soc. Chem. Ind.*, 1948, **67**, 121-125. (The phosphate in potato starch is

esterified with the amylopectin, while the phosphatides of wheat starch are adsorbed by the amylose.) *C.A.*, **42**, 8005.

285. L. H. LAMPITT, C. H. F. FULLER and N. GOLDENBERG, 'The fractionation of potato starch. 6. The retrogradation of potato starch fractions', *J. Soc. Chem. Ind.*, 1948, **67**, 179-184. (The cold-water-soluble and hot-water-soluble fractions from ball-milled potato starch exhibit retrogradation tendencies similar to the corresponding fractions from wheat starch.) *C.A.*, **43**, 427.

286. L. LEHRMAN, 'Fatty acids associated with rice starch', *J. Am. Chem. Soc.*, 1929, **51**, 2185-2188. (The fatty acids are identified as palmitic (36 per cent. of lipid material), oleic (35 per cent.) and linoleic (29 per cent.).) *C.A.*, **24**, 1759.

287. L. LEHRMAN, 'Fatty acids associated with wheat starch', *J. Am. Chem. Soc.*, 1930, **52**, 808-811. (The fatty acids are palmitic (35 per cent. of total lipid), oleic (41 per cent.) and linoleic (24 per cent.).) *C.A.*, **24**, 2323.

288. L. LEHRMAN, 'Fatty acids associated with cassava starch', *J. Am. Chem. Soc.*, 1932, **54**, 2527-2530. (Finds palmitic, oleic, linoleic and linolenic acids.) *C.A.*, **26**, 4200.

289. L. LEHRMAN, 'Comment on "Absence of combined fatty acid in cereal starches"', *J. Am. Chem. Soc.*, 1939, **61**, 212-213. (Criticises Schoch's statement that fatty acid of corn starch is extraneous.) *C.A.*, **33**, 5220.

290. L. LEHRMAN, 'Nature of the fatty acids associated with starch. The adsorption of palmitic acid by potato and defatted corn and rice starches', *J. Am. Chem. Soc.*, 1942, **64**, 2144-2146. (Adsorption of fatty acid from methanol medium follows a typical Freundlich isotherm.) *C.A.*, **36**, 6836.

291. L. LEHRMAN, 'The extraction of fat and phosphorus from wheat starch', *J. Am. Chem. Soc.*, 1945, **67**, 1541-1542. (Extraction of wheat starch with 80 per cent. dioxane removes all the fat and most of the phosphorus. A small residual portion of the phosphorus appears to be linked with the starch.) *C.A.*, **39**, 5527.

292. L. LEHRMAN and E. KABAT, 'Absence of fatty acids associated with potato starch', *J. Am. Chem. Soc.*, 1933, **55**, 850. *C.A.*, **27**, 1779.

293. L. LEHRMAN and E. A. KABAT, 'Fatty acids associated with banana starch', *J. Am. Chem. Soc.*, 1937, **59**, 1050-1051. (Banana starch contains 0·2 per cent. combined fatty acid, together with a small amount of phytosterol.) *C.A.*, **31**, 6045.

294. W. W. LEPESCHKIN, 'The verification of chemical reactions in the pasting of starch', *Kolloid-Z.*, 1942, **101**, 196-198. (Gelatinisation of the starch granule is initiated by a reaction between water and the starch polysaccharides.) *C.A.*, **38**, 6591.

295. I. MARKEVICH and M. KHOLODOVA, 'Effect of the time of storage of potatoes on the capacity of the potato starch and amylopectin to form colloidal compounds with proteins', *Colloid. J.* (U.S.S.R.), 1938, **4**, 387-399. (Storage of potatoes at 0·5° C. decreases the capacity of the starch to bind proteins.) *C.A.*, **33**, 2751.

296. K. H. MEYER, 'Branched and unbranched starch components', *Naturwissenschaften*, 1940, **28**, 397. (See Reference 53.) *C.A.*, **35**, 3843.

297. K. H. MEYER, 'Branching and net formation in amylopectin molecules', *Naturwissenschaften*, 1940, **28**, 722. (See Reference 59.) *C.A.*, **35**, 4264.

298. K. H. MEYER, 'The constitution of amylopectin', *Naturwissenschaften*, 1940, **28**, 564-565. (Discussion of alternate treatment of amylopectin with beta-amylase and alpha-glucosidase, see Reference 54.) *C.A.*, **35**, 4353.

299. K. H. MEYER, 'Terminal group determination of starch components. Remarks on the papers of the same name by K. Hess and B. Krajnc, and by Hess and Steurer', *Ber.*, 1940, **73B**, 1298-1299. (Review and discussion.) *C.A.*, **35**, 5904.

300. K. H. Meyer, 'The constitution of amylose and amylopectin in corn starch', *Compt. rend. soc. phys. hist. nat. Geneve*, 1940, **57**, 19-23 (in *Arch. Sci. phys. nat.*, 1940, **22**, Mar.-Apr.). (Review of the preparation, viscosity data and film-forming properties of amylose and amylopectin). *C.A.*, **36**, 5049.

301. K. H. Meyer, 'Maize starch: Composition', *Arch. Sci. phys. nat.*, 1940 (5), **22**, Suppl. 19-23. (Discussion.)

302. K. H. Meyer, 'The arrangement of the glucose residues in glycogen', *Naturwissenschaften*, 1941, **29**, 287. (The branch lengths of glycogen and amylopectin are 11 and 25 glucose units, respectively. Methylation studies on the residual dextrin from amylopectin indicate that 15-18 glucose units of the branch are outside the point of branching and hence available to beta-amylase, the remaining 8-9 glucose units are protected inside the branch point.) *C.A.*, **37**, 91.

303. K. H. Meyer, 'New investigations of starch', *Melliand Textilber.*, 1943, **24**, 125-129. (Review.) *C.A.*, **37**, 6486.

304. K. H. Meyer, 'Starch and glycogen', *Anales Fis. y Quim.* (Madrid), 1947, **43**, 287-298. (Review.) *C.A.*, **41**, 6743.

305. K. H. Meyer, 'Constitution and structure of starch and its enzymatic synthesis and degradation', *Osterreichische Chem-Zt.*, 1949, **50**, 46-52. (Review.)

306. K. H. Meyer and P. Bernfeld, 'Kinetics of degradation of starch by wheat amylase', *Arch. Sci. phys. nat.*, July-Aug., 1940, **22**. (The rate of beta-amylase degradation of starch should remain constant until the molecule is completely broken down or until a branch point is reached.) *C.A.*, **35**, 6862.

307. K. H. Meyer and P. Bernfeld, 'Starch. 14. Iodine color reaction with starch and glycogen', *Helv. Chim. Acta*, 1941, **24**, 389-393. (The shade of colour of the iodine complex is related to chain length.) *C.A.* **35**, 7377.

308. K. H. Meyer and P. Bernfeld, 'Starch. 19. Degradation of amylose by alpha-amylase', *Helv. Chim. Acta*, 1941, **24**, 359-369 E. (Degradation of amylose by alpha-amylase is random, though the larger molecules are preferentially attacked.) *C.A.*, **36**, 5376.

309. K. H. Meyer and P. Bernfeld, 'Starch. 22. The action of potato phosphorylase', *Helv. Chim. Acta*, 1942, **25**, 404-405. (Unlike yeast phosphorylase, potato phosphorylase does not attack the α-1, 6 linkage in limit dextrin.) *C.A.*, **36**, 4834.

310. K. H. Meyer and P. Bernfeld, 'Recent investigations on starch', *Melliand Textilber.*, 1948, **29**, 165-168. (Review.)

311. K. H. Meyer, P. Bernfeld, P. Gurtler and G. Noelting, 'Starch. 39. Corn starch', *Helv. Chim. Acta*, 1948, **31**, 108-110 (cf. Reference 105). *C.A.*, **42**, 3334.

312. K. H. Meyer, P. Bernfeld and J. Press, 'Starch. 8. Degradation of the constituents of starch by beta-amylase', *Helv. Chim. Acta*, 1940 **23**, 1465-1476. (Action of beta-amylase on starch is not materially altered by prior hypoiodite oxidation of terminal aldehyde; the point of enzyme attack must therefore be the non-aldehydic terminus. Incomplete conversion of amylose by beta-amylase is attributed to preferential hydrolysis of shorter chains and aggregation of the longer chain components.) *C.A.*, **35**, 3117.

313. K. H. Meyer and W. Brentano, 'Corn starch', *Arch. Sci. phys. nat.*, July-Aug., 1936, **18**. (Centrifugation of 1 per cent. corn starch pastes gives soluble amylose and insoluble amylopectin. The latter has a 3-dimensional mesh structure.) *C.A.*, **31**, 6044.

314. K. H. Meyer and M. Fuld, 'Starch. 12. Arrangement of glucose residues in glycogen', *Helv. Chim. Acta*, 1941, **24**, 375-378. (See Reference 302.) *C.A.*, **35**, 7376.

315. K. H. Meyer and M. Fuld, 'Starch. 17. The starch of glutinous rice', *Helv. Chim. Acta*, 1941, **24**, 1404-1407. (Glutinous (waxy) rice starch

is similar to glycogen, giving a red colour with iodine, yielding 40 per cent. beta-amylase residual dextrin, and containing 6 per cent. terminal groups.) *C.A.*, **36**, 4365.

316. K. H. MEYER and M. FULD, 'Starch. 20. Viscosity of starch pastes', *Helv. Chim. Acta*, 1942, **25**, 391-398. (The high viscosity of potato starch pastes is attributed to the behaviour of the granules as liquid-filled vesicles. The granules of other starches disintegrate on cooking.) *C.A.*, **36**, 5048.

317. K. H. MEYER and MARIA FULD, 'Starch. 17. The starch of glutinous rice', *Helv. Chim. Acta*, 1941, **24**, 1404-1407. (Waxy maize starch gives a red iodine colour, yields 40 per cent. of limit dextrin with β-amylase, and contains approximately 6 per cent. of terminal end-groups.)

318. K. H. MEYER and MARIA FULD, 'Starch. 44. The glycogen of *Zea mays*, var. Golden Bantam', *Helv. Chim. Acta.*, 1949, **32**, 757-761. (Periodate end-group assay and β-amylase convertability of corn glycogen are identical with values for animal glycogen.)

319. K. H. MEYER, P. GURTLER and P. BERNFELD, 'Structure of amylopectin', *Nature*, 1947, **160**, 900. (Discussion in support of an irregularly branched structure for amylopectin.)

320. K. H. MEYER and P. HEINRICH. 'Starch. 23. Composition of starch from potato leaves, tuber sprouts and tubers', *Helv. Chim. Acta*, 1942, **25**, 1038-1046. (Starches were isolated from the leaves, sprouts and tubers of the potato plant. Amylose contents were calculated to be 17 per cent., 46 per cent., and 18 per cent. respectively.) *C.A.*, **37**, 1153; incorrectly abstracted.—T. J. S.

321. K. H. MEYER and P. HEINRICH, 'Composition of several kinds of starch', *Helv. Chim. Acta*, 1942, **25**, 1639-1650. (Fractionation of sago, tapioca, pea and waxy maize starches. The latter contains no amylose.) *C.A.*, **37**, 6151.

322. K. H. MEYER, E. PREISWERK and R. JEANLOZ, 'Starch. 15. Kinetics of the degradation of undegraded potato and corn starches by β-amylase', *Helv. Chim. Acta*, 1941, **24**, 1395-1400. (Effect of enzyme concentration and temperature on the rate of β-amylase action.) *C.A.*, **36**, 4365.

323. K. H. MEYER and J. PRESS, 'Starch. 9. Degradation by β-amylase and the mass action law', *Helv. Chim. Acta*, 1941, **24**, 50-58. (Kinetics of the β-amylase degradation of starch and amylose.) *C.A.*, **35**, 4783.

324. K. H. MEYER and J. PRESS, 'Starch. 10. Degradation of glycogen by β-amylase', *Helv. Chim. Acta*, 1941, **24**, 58-62. (Kinetics of the β-amylase degradation of glycogen.)

325. K. H. MEYER and P. RATHGEB, 'Starch. 45. Determination of end-groups in polysaccharides and oligosaccharides by means of periodate', *Helv. Chim. Acta*, 1949, **32**, 1102-1107. (Recommends that the oxidation be conducted at 0° C. and a *p*H of 4·2-5·8.)

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Iowa State College J. Science, 1947, **22**, 58-60. (See Reference 113.)

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show no adsorptive capacity for carbohydrates.) *C.A.*, **29**, 4782 (Note : Compare with butanol fractionation.—T. J. S.)

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The aging of starch solutions from the colloid-chemical, enzymic and X-ray spectrum point of view', *Kolloidchem-Beihefte*, 1939, **49**, 455-470. (Ageing of solutions of various starch fractions and modified starches was followed by turbidity, viscosity, iodine colour, conductivity and X-ray spectrum.) *C.A.*, **33**, 6683.

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and glutinous rice starch. 3', *J. Faculty Sci. Hokkaido Imp. Univ.* (3), 1934, **2**, 59-79. (Compares the viscosity, silica content and phosphorus content of several varieties of waxy rice starch. The latter is claimed to have a higher amylose content than non-waxy rice.) *C.A.*, **29**, 2013. (Note: it appears that this writer uses the terms amylose and amylopectin in a reversed sense.—T. J. S.)

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presence of such a group, as it would tend to
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FOUR theories havses were also considered to contribute to this
of the phosphorus
described in Chapter amylopectin from potato starch, and after
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briefly. ed in some respects from the amyloses of

The Formation oduct[17] was then re-esterified according to
regards a clear starch sorg[20, 21] by treating it with phosphorus
colloidal particles consist ice of calcium carbonate. The resulting
molecules and that the systeopears to lose its calcium ions on
According to this worker, the n. product closely resembling the
complexes around the phosphate an enhanced viscosity and a
pensated by hydrogen, alkali or phosphorus-content was raised
complexes have the power of forming st. by this method, and the
these complexes yet higher ones again, thriour of the synthetic
turally occurring

$$(MA)\mp C_x \mp \rightarrow [(MAC)_y(MA)] \pm C_x + \rightarrow [(MAC_x)_y(MA)C_x]_z(MAC_x)_y(MA] \pm$$

the amylo-
on a

these representing complexes of the first, second and third orders respectively, where M is the non-electrolyte portion, A the phosphate ion, and C a cation. Thus the phosphate ion would be masked entirely by the carbohydrate and the cation partially so.[4] It follows that removal of phosphorus can only be accomplished by previously destroying the micelle or complex to liberate the phosphate ion, but Samec[5] has shown that the phosphoric acid may be removed by heating with water under pressure, followed by electrodialysis of the solution, although the degree of dispersion is not changed appreciably.

By repeated washing with 1 per cent. hydrochloric acid, Samec was successful in removing 94 per cent. of the cations present, and has also shown that the hydrogen ions in potato starch are not masked.

The Adsorption Theory.—J. Janicki[8] considers that the major portion of the phosphorus in starch is adsorbed on the starch in the form of colloidal phosphates, although from 0·0 to 9 per cent. of the total phosphorus may be held as an ester. He considers that changes of viscosity of starch solutions on ageine

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ly held than the cations. How-
e ion is not adsorbed from solution.

Acid Theory.—If the starch is broken
r, the phosphorus-content is still retained.
nd his co-workers, used pancreas extract to
the hydrolysis. T. Posternak[9] obtained a biose-
horic acid, whilst Northrop and Nelson[11] obtained a compound of low molecular weight containing 5 per cent. phosphorus from potato starch, a similar compound being obtained by Samec,[12] who also peptised starch by the action of ultra-violet light and obtained a product containing 3·26 per cent. P_2O_5. Lampitt and co-workers[13] from their work on ball-milled wheat starch have shown that a phosphorus-rich fraction is present. M. Samec and F. von Hoefft[14] postulated the presence of a dibasic amylophosphoric acid in starch in which the amylose and the phosphoric acid are connected through an ester linkage and in this Posternak[15] agrees. He assigns the formula

$$\begin{array}{l} | \\ \mathrm{St}-\mathrm{O}-\mathrm{PO}< \begin{array}{l} \mathrm{OR_1} \\ \mathrm{OR_2} \end{array} \\ | \end{array}$$

in which the St. is a glucose unit in the starch chain and R_1 and R_2 are metal or hydrogen atoms. This compound is hydrolysed by heating with water under pressure, and the longer the duration of heating the greater the loss of phosphoric acid by the amylopectin.[11]

The colloidal behaviour of amylopectin would be greatly in-

fluenced by the presence of such a group, as it would tend to increase the ionic hydration of the micelles and thus lead to an enhanced viscosity in solution as compared with the phosphorus-free carbohydrate. When the phosphoric acid is split off, the viscosity of the solution falls and the number of micelles travelling to the anode when a potential is applied is decreased. Thus, the hydrolysis of the ester is considered by these workers to be a fundamental cause of the fall in the viscosity of the solution, but other minor causes were also considered to contribute to this effect.[16–19]

Samec[14] separated amylopectin from potato starch, and after saponifying it to remove the phosphorus-content, obtained a product which differed in some respects from the amyloses of Maquenne. This product[17] was then re-esterified according to the method of Neuberg[20, 21] by treating it with phosphorus oxychloride in the presence of calcium carbonate. The resulting calcium amylophosphate appears to lose its calcium ions on electrodialysis,[22] and gives a product closely resembling the original amylopectin, except for an enhanced viscosity and a higher phosphorus-content. The phosphorus-content was raised from 0·0 per cent. to above 2 per cent. by this method, and the acidic nature and electrochemical behaviour of the synthetic amylopectin were similar to those of the naturally occurring product in starch. J. B. Kerb[23] has also prepared the amylophosphoric acid by the action of phosphorus oxychloride on a suspension of amylose and calcium carbonate in chloroform.

Samec[24] was also able to obtain similar results with amylopectin derived from different starches, whilst H. Pringsheim[25] also obtained a gelatinous phosphoric ester by the action of phosphoryl chloride, in the presence of pyridine, on the polyamyloses obtained by the action of *Bacillus macerans* on starch.

The amylophosphoric acid prepared from potato starch gives a practically transparent jelly similar to the potassium salt which, according to H. Tryller,[26] occurs in the granule. If the starch is washed with hard water, the potassium is replaced by the calcium in the water. The barium and calcium salts give cloudy pastes with water, as they have but very slight solubility, and the pastes are therefore much less viscous than those obtained from the potassium salt or the free acid.

The synthetically prepared amylopectins of the cereal starches are acid and resemble potato and tapioca amylopectins in behaviour.

Samec[16] noted that a decrease in the viscosity of a starch solution was accompanied by an increase in its conductivity. The same

changes that take place on ageing occur on heating, but at a much greater rate. As certain physical properties, e.g. optical rotation of the solution, do not alter in such cases, the changes cannot be due to degradation by hydrolysis or to peptisation.

Concurrently with the fall in the viscosity of starch solutions on ageing or on heating, a decrease occurs in the amount of material that can be precipitated by alcohol, and also a corresponding decrease in the amount of substance migrating under the action of electric current. Demineralisation by freezing and thawing of starch solutions leads to the same effect as ageing or heating under pressure. A 1 per cent. potato-starch solution made by heating starch under pressure has a high initial viscosity which is decreased if acid is added. The addition of a small amount of alkali causes a slight increase in the viscosity, but as the concentration of alkali in the solution is increased, the viscosity falls to a minimum and then rapidly increases again.

From potentiometric measurements Samec[24] has obtained characteristic curves connecting the *p*H of the solution and the amount of alkali added. During the neutralisation of the first hydrogen atom the viscosity increases slightly at first, decreases to a minimum at a concentration of one equivalent of alkali corresponding to 1 gm.-equivalent of phosphorus, and on continued addition of alkali the viscosity increases in a discontinuous manner with the increase in *p*H value. The curves for sodium carbonate and sodium hydroxide are similar in character.

From these facts Samec therefore concluded that the solution of amylo-phosphoric acid has a structure consisting of hydrated jelly-like particles, which increase in size owing to increased hydration on the addition of the first portion of the alkali with a consequent increase in viscosity of the solution. When the first hydrogen atom is neutralised, the particles pass into colloidal solution, and then the hydration of the dispersed phase increases as the addition of the alkali is continued. Thus the decrease in the viscosity to a minimum at the first end-point and the subsequent increase in viscosity may be explained.

On ageing, at constant temperature, the ratio between the initial and final viscosities is the same for solutions containing different concentrations of starch, and once the change has taken place the original viscosity cannot be restored by further treatment of the solution.

Richardson and Higginbotham[27] have studied the influence of the combined phosphoric acid on the swelling of granular starch. They point out that in some starches, e.g. potato, the phosphoric acid exists in the starch as an ester but consider that in other

starches, e.g. wheat, part of the phosphorus-containing impurity is removable by extraction with cold dilute acid or methanol (see O. Rask[28] and Schoch[29]).

The metals combined with the phosphoric acid play an important part in swelling phenomena, for example, the swelling ability and the viscosity of a 3 per cent. paste of sodium starch are greater than those of pastes made from the corresponding calcium starch. In the presence of salt, however, these differences are not observed, and the above workers suggest this is due to the greater tendency of the sodium atoms to ionise and diffuse into the water. They found no simple correlation between the phosphorus-content of starches and the swelling behaviour after washing with the same salt solution. If the average chainlengths of the starch molecules were the same[30] as they were in eight starches examined, such a correlation might be expected. They were therefore led to the conclusion that there may be differences in sub-microscopical structure which would mask the effect of the phosphorus on the swelling in pure water.

From the foregoing we see that the amylophosphoric-acid theory accounted for the mobility of the cations and the retention of phosphorus on washing, the chemical reactivity of the unswollen granules, and a number of other observations, although Myrbäck and Ahlborg[31] consider it unlikely that there is any carbohydrate-phosphorus complex in starch at all. The weight of evidence, however, is against this, as will be seen in the work discussed in other Chapters.

Karrer[32, 33] considers that the phosphorus in potato starch is distributed through all fractions of the starch, and is supported in this by Taylor and Schoch,[34] but Baldwin[35] and Samec[36] maintain that it is held in the amylopectin portion. Later Samec[37] prepared amylophosphoric acids by a number of different methods, such as washing potato and similar starches with acids, prolonged heating of starch pastes, severe grinding, exposure to ultra-violet light, and suitable hydrolysis by diastase. In the solutions thus prepared he believed that amylophosphoric acids are present having micelles of different sizes and different phosphorus-content, and further, that the paste formation depends upon having a proper balance between the tendency to associate and to hydrate. Malfitano and Catoire[38] and other workers[39] also place considerable significance on the phosphorus content of the various alleged amyloses.

To sum up the present position: Samec[40] considers the presence of phosphorus, in the form of an amylophosphoric ester, is partially responsible for the viscometric behaviour of starch,

and some force is lent to his views by the discovery by Richardson and Higginbotham[27] that the degree of swelling of different sized starch granules can be correlated with the phosphorus content of potato but not wheat starch. Other workers[41-44] attribute the viscosity of starch pastes to the relative volume of the gelatinised granules in the paste. More work is required before a theory is put forward to cover the viscometric behaviour of all starches.

The Significance of Nitrogen in Starch.—Both wheat and potato starches can be freed from cations by treatment with dilute hydrochloric acid, followed by a thorough washing with water, and from the sols of starches treated in this manner the amylose and amylopectin fractions can be obtained. The properties of the amylose fractions from the various starches are very similar, but a comparison of the properties of the different amylopectins show that distinct differences exist in their behaviour.

The starch of gramineæ is relatively rich in phosphorus, and after a very careful purification of the amylopectin fraction and an estimation of the phosphorus present in it, some measure of its conductivity and hydrogen-ion activity in solution should be predictable. In practice, however, the values obtained do not correspond to those which might be expected if the amylophosphoric-acid theory is correct. In attempting to explain the divergency, Samec estimated the phosphorus and nitrogen in both wheat and potato starches, using samples that had been exhaustively purified, and from his results he infers that in wheat starch the amounts of phosphorus and nitrogen exist in definite stoichiometric proportions. Further, he found that in certain solutions of wheat-starch amylopectin, this substance possessed a negative charge, whilst in other fractions there was a tendency for it to migrate to the cathode.

Samec suspected at first the presence of phytovitelines, which are thought by some workers to contain phosphorus. These compounds are soluble in ammonia, so Samec then treated the wheat-starch amylopectin with ammonia in order to remove these products, if present. The treatment did not remove any phosphorus, the negative charge on the amylopectin was found to have increased, the substance itself resembled the amylopectin prepared from potato starch more closely than before the treatment, and the viscosity of its solutions had increased.

The results obtained by Samec thus point to the absence of phytovitelines, and it appeared feasible that the nitrogen was present as protein matter. He, therefore, treated wheat-starch amylopectin with pepsin and hydrochloric acid, or merely with potassium hydroxide solution to remove any proteins present, and

obtained products possessing the physical properties and acidity resembling those of potato-starch or tapioca-starch amylopectins.

Samec[12, 45] explains the differences between the physical properties of gramineæ amylopectins and those obtained from tuber starches by suggesting that the former is a protein-amylo-phosphoric complex in which the protein inactivates the phosphorus of the amylopectin electrochemically, and also to some extent chemically, whilst the tuber amylopectins are metallic salts of amylophosphoric acid. Posternak[15] and Nottbohm and Mayer[46] consider the phosphorus and nitrogen to be present as a phosphatide, and the latter workers find some 70 per cent. of the phosphatide content of wheat flour (calculated from the choline content) to be present as an adsorbate in starch. As they, and other workers,[47–49] point out, phosphatides can form stable complexes with carbohydrates, and thus wheat starch may possibly contain a carbohydrate-phosphatide complex, a conclusion supported by Samec in further papers.[50–54] In conjunction with Waldschmidt-Leitz[55–56] he found that amylophosphatase from ungerminated barley liberates phosphorus from potato but not wheat starch, free reducing groups being formed concurrently in the former. With kidney phosphatase, free from amylase, both starches are dephosphorylated, accompanied, in the case of wheat starch, by the liberation not of reducing groups but of free amino groups, thus showing the close connection between the phosphorus and nitrogen present in wheat starch.

The Coacervation Theory.—Samec first suggested that the protein was bound 'salt-like' to the amylophosphoric acid, but later he[57] himself showed this view to be unsatisfactory. P. Koets[58] suggests that coacervation between the protein and the amylophosphoric acid takes place. Coacervation is the name given to the separation of droplets from solutions of hydrophilic colloids caused by the difference in sign of two colloids present, causing the micelles to approach, with the result that their double layers partially discharge each other. The more loosely-bound water of hydration of the micelles is lost when the partial discharge takes place, and when the force of attraction between the micelles, due to the difference in sign, is balanced by the resistance to displacement of the more rigidly bound water of hydration. A conglomerate, consisting of positive and negative micelles which remain together but, as individuals, still retaining a portion of their water of hydration, is thus obtained.

If this theory is correct it might be expected that potato amylopectin would move cataphoretically to the anode, as the negatively charged amylophosphoric acid is present to a greater extent than

the positively charged protein, giving a resultant negative charge. In practice this is observed. Samec and Antonovic[59] have shown that excess amylophosphoric acid was present in certain fractions of wheat amylopectin which moved to the anode, whereas in other fractions in which the protein was in excess there was a tendency to move to the cathode.

Koets points out that the amylopectin made by Samec was prepared by removing the phosphoric acid from a sample of amylopectin and then re-esterifying the amylose so obtained. Amylopectin prepared by the esterification of pure amylose, however, does not show the high viscosity of the amylopectin prepared by Samec's method, and is, indeed, little more than that of the amylose from which it was prepared. This, according to Koets, may probably be due to the presence of a small amount of protein matter in Samec's original amylopectin which was liberated when the phosphoric acid was removed, but which was still present in the amylose obtained, so that when this was re-esterified a fresh coacervate was formed between the amylophosphoric acid formed and the protein matter present. It has also been shown[26, 50, 58–61] that by increasing the amount of positively charged protein matter coacervating with the amylophosphoric acid from potato starch, a product is obtained which closely resembles the amylopectin obtained from wheat starch, which is known to contain a much higher proportion of protein matter.

Samec found that the colour given by an iodine-potassium iodide solution with amylopectin passes from blue to red the greater the nitrogen-content of the sample, and Koets finds that the same behaviour is shown by coacervates of amylophosphoric acid and protein, so that the facts fit in quite well with the theory.

From viscosity measurements in the presence of sodium hydroxide, Koets and H. R. Kruyt[62–63] conclude that the OH-groups at the surface of the amylose micelles dissociate and function as centres of electric charges and hydration, the extent of dissociation being governed by the *p*H value of the solution. In neutral or weakly acidic solutions they consider the dissociation and surface charges to be very small, neither being strong enough to overcome the crystallite cohesion of the molecules.

In alkaline media the dissociation, and therefore the density, of the surface charge increases, leading to the formation of a double surface-layer about the particles, and attaining a maximum at approximately 0·1 N caustic soda. In contrast with other lyophilic colloids, e.g. gum-arabic, which contain highly ionised surface groups, a higher *p*H value is necessary in the case of

amylose to bring this about. As the amount of sodium hydroxide is increased, an exchange takes place between the H and Na ions, and the addition of a small amount of alkali causes the solution to gel owing to surface dehydration of the swollen micelles. This takes place in solutions of 1·5-3 N alkali. At concentrations above 3·5 N the alkali itself brings about dehydration. Amylophosphoric acid possesses an electric layer which increases with the number of phosphoric-acid groups introduced. A mixture of amylophosphoric acid and amylose reacts with the positive component of proteins to form a complex, but amylose alone does not have this property.

Samec's explanation is considered to be unsatisfactory by W. N. Haworth and E. L. Hirst.[64]

Fatty Acids Present in Certain Starches.—In 1885 L. Sostegni[65] hydrolysed rice starch with hydrochloric acid, and noted that the insoluble portion of the starch remaining after this treatment contained a substantial amount of an ether-soluble fatty material which melted at 47-48 C. Ren-Ichiro Aoi[66] examined rice starches made both by the Japanese and by the Chinese methods, and found 0·56 and 0·66 per cent. of fatty matter respectively.

Further work followed and is described by Dr. Schoch in Chapter 6 so that we do not need to make more than passing mention here of the existence of fatty acids in certain starches.

Other Acids Present in Starch as Esters.—Certain commercial maize starches have acid properties, and these are ascribed by Samec[67] to the presence of a sulphonic-acid group, which he thinks may be introduced into the molecule when the manufacture of starch involves the purification or bleaching with sulphurous acid. He finds that starch may be readily sulphonated with sulphuryl chloride, and that the product has acid properties similar to those acquired when the phosphoric ester is formed.

The acidity of roast dextrins,[68] soluble starches,[69] and starches that swell in cold water,[70] is ascribed by Samec to the formation of carboxyl groups in the carbohydrate molecule, especially in those processes which involve the use of oxidising agents. The introduction of carboxylic acid groups into starches of low phosphorus content by oxidation enhances the swelling of the granular starch. J. A. Radley has noticed that the acidity of roast dextrin appears to increase a little after the dextrin has been re-moistened, which may be due to acid formed from anhydrides present, or to oxidation of aldehydic compounds formed during the roasting process.

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Chapter 8

STARCH RETROGRADATION

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Retrogradation is a term given by early workers[1–12] to that group of phenomena occurring during the normal aseptic ageing of an aqueous starch paste. More specifically, the term has been employed to designate the spontaneous precipitation of starch from solution. The word retrogradation was selected because the starch appeared to be reverting to its original condition of cold water insolubility.[3] Prior to precipitation of the starch, the solution becomes opalescent, increases in cloudiness, increases in resistance to enzyme action and decreases in viscosity. These phenomena are considered to arise from the tendency of suspended or dissolved starch molecules or groups of molecules to unite with one another to form particles of increasing size. Thus, in a sense, retrogradation represents an attempt at crystallisation on the part of large unwieldy molecules.

The term retrogradation is limited in this chapter to the designation of only those reactions and effects which occur spontaneously during the aseptic ageing of a starch solution, starch paste, or starch water mixture. It is believed that the term can serve a definite need in starch chemistry only if its use be restricted to those reactions and effects which necessarily result from the inherent tendency of starch molecules to bond with one another by secondary forces and which eventually lead toward the semi-crystalline state characterised by a 'B' X-ray pattern. Consequently, the rapid precipitation of starch from solution by the use of alcohol, acetone, etc., the precipitation of starch as a starch-protein complex, or the precipitation of amylose as a complex with any of the starch fractionating agents (see Chapter 7), are not considered here as retrogradation phenomena.

Considerable practical importance is attached to retrogradation. It is believed to be a factor contributing to the staling of bread and to the changes which occur in the texture of canned cheese at peas, corn and other foods containing high starch concentrate out[17–19] It is also of importance in the textile, paper and adhesive

where changes in prepared batches of pastes are highly undesirable. In these instances the formation of a 'skin' on the size bath, the formation of a precipitate in the paste or its solidification to a gel causes the paste to be difficult to handle on machines and may cause it to be applied in an uneven manner. In the industry, this transformation of a paste to a gel is called 'set-back'.

Most of the interest among those working on retrogradation has been on the acceleration, retardation or prevention of retrogradation either through the use of chemicals or by the use of physical means such as temperature. Much of the published work on retrogradation is incomplete, and often the reported data are based on questionable analytical techniques. As yet, few accurate quantitative studies have been published on the comparative rates and extents of retrogradation of various common starches or starch fractions. The majority of retrogradation studies have been made with potato starch, although some work has been done on wheat[7, 12, 15, 19, 19a, 21, 26] corn[12, 19, 19a, 20, 23, 24, 26] rice,[12] barley,[14] and sago[19] starches. Often the starches used for these studies were in a degraded condition. It is established,[19c] however, that different starches retrograde at different rates and to various degrees (Fig. 8 : 1). These curves show the rate at which insoluble material is precipitated from 2 per cent. starch suspensions allowed to stand under aseptic condition at 0-2° C. The suspensions are prepared by autoclaving the starch in water for several hours and centrifuging the hot solution.

Factors Affecting Retrogradation.—The rate and extent of starch retrogradation is dependent on both physical and chemical factors. The most prominent factors affecting retrogradation are temperature, concentration of the starch paste, molecular size and shape of the starch molecules, hydrogen-ion concentration and the presence of other chemical agents in the paste. These various factors will be discussed in order, except for the effect of the shape of the starch molecule which will be considered in connection with the comparative behaviour of amylose and amylopectin.

Leon Maquenne was the first worker to investigate the spontaneous precipitation of starch from aqueous solutions and to examine the effect of temperature on the rate of precipitation.[1-6] In general, Maquenne employed 5 per cent. potato starch pastes which were autoclaved at 120° C. for 15 minutes or at 130° C. for 0 minutes. Pastes prepared by either autoclave treatment aved in the same manner. The extent of retrogradation was ed by measuring the decrease in the amount of starch fied by malt diastase. This was possible since it was t retrograded starch is not attacked by diastase.[1, 4]

Comparison of starch pastes at the temperatures of 36°, 22°, 14° and 0° C. showed that the rate of retrogradation increases rapidly as the temperature is lowered. When a starch paste of 5 per cent. concentration was held at 0° C., it was observed that an appreciable amount of retrograded starch could be detected within an hour,[4] that three-quarters of the total observed retrogradation took place within 3 days and that retrogradation was complete in 9 days.[2] Using a similar analytical technique but substituting ptyalin for malt diastase, Sallinger[14] found that a solution of Lintner potato starch retrogrades rapidly at 7° C. In working with solutions of a wheat starch,[15] which had been partially

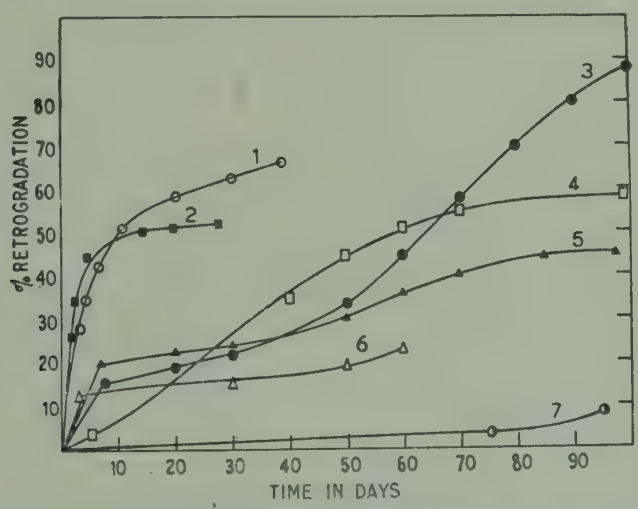

FIG. 8: 1.—Rate of retrogradation of various common starches from 2 per cent. aqueous dispersions. 1, corn; 2, wheat; 3, white potato; 4, sweet potato; 5, arrowroot; 6, tapioca; 7, waxy corn.

degraded by long grinding in a ball mill, recent workers have noted that very little retrogradation takes place at 37° to 62° C. but that an extensive retrogradation occurs at 4° C. Similar results were obtained by Maquenne with 5 per cent. potato starch pastes.[5] Katz and Weidinger[16] have also observed that no retrogradation occurs above 60-70° C.

As the temperature is lowered toward freezing, the rate of retrogradation continuously increases.[19b] This is partially illustrated in Fig. 8 : 2 which shows a comparison of the rate of retrogradation or precipitation of wheat amylose from aqueous solutions of 0·85 per cent. concentration at 0° C. and at 25° C. The extent of retrogradation is also affected by temperature, being greater at lower temperatures.

Freezing of a starch solution causes the starch to separate out[17–19]

...us and flocculent coagulum. and to remain, after thawing, as a fibastes are frozen, thawed and When 2·5 per cent. corn starch, 3 per cent. of the starch remains warmed to 90-100° C., about 90 one way, potato starch yields about insoluble.[20] Treated in the sa...hile tapioca and waxy corn starches 80 per cent. insoluble matte...[20] Corn and wheat starch pastes of produce no insoluble mat...centration remain, after thawing, as a 5 per cent. or more ... This sponge has been suggested for spongy mass.[18, 21] ...medical dressing,[23] or, when dried, as a constituent of food... or candies.[24] Slow freezing of the solution is said to produce larger aggregates of coagulum than rapid freezing.[25]

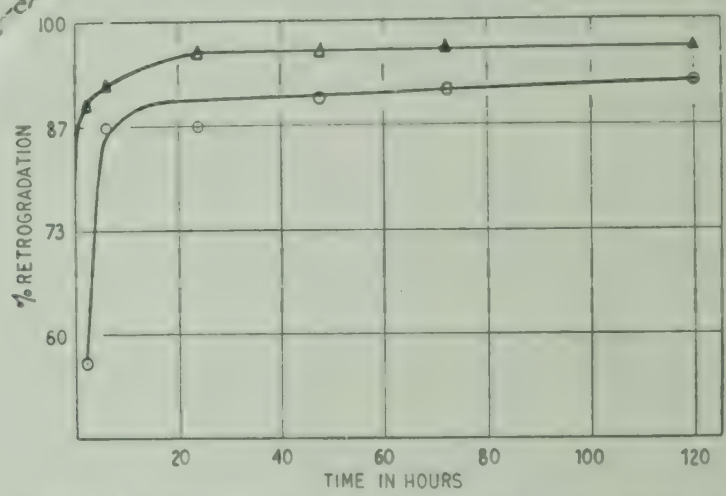

FIG. 8:2.—Rate of retrogradation of wheat amylose from aqueous solution of 0·85 per cent. concentration. Δ at 0° C.; (·) at 25° C.

Furthermore, the properties of the thawed gel depend upon the temperature to which it is cooled. Spongy gels thawed from solutions frozen at —70° C. are more slimy than gels from solutions frozen at —2° C.

The rate of retrogradation increases with increasing concentration of the starch solution.[5, 13] In a series of starch pastes allowed to stand at 9° C. for 4 days, Maquenne[5] has found that the amount of deposition is increased markedly by relatively small changes in concentration of the paste. The effect of concentration on the rate of retrogradation is brought out clearly by a series of studies[19b] made with corn amylose (Fig. 8 : 3). Whole starch and amylopectin both of which retrograde more slowly than amylose, are somewhat less affected by concentration changes.

The average size of the molecules plays an important role in the determination of both the rate and extent to which a starch

retrogrades. Roux[9] has noticed a decreased rate of retrogradation for starch heated in water to 125°-150° C., and, similarly, Katz[28] has noticed a decrease in the rate of retrogradation for starch partially dextrinised by heating in the dry state to temperatures above 100° C. Both treatments may be presumed to cause a lowering of the average molecular weight of the starch. A decrease in molecular size of the starch retrogrades. Thus, in a series of solutions of acid hydrolysed starches those apparently lowers, also, the extent to which starches of highest reducing power and consequently of lowest average molecular weight are observed to retrograde to the smallest extent.[19, 29, 30] Experiments of Staudinger and Husemann[29] have led them to the opinion that the extent of retrogradation decreases in proportion to the decrease in molecular weight of the starch. The manner in which molecular size controls the rate of retrogradation of amylose is described later

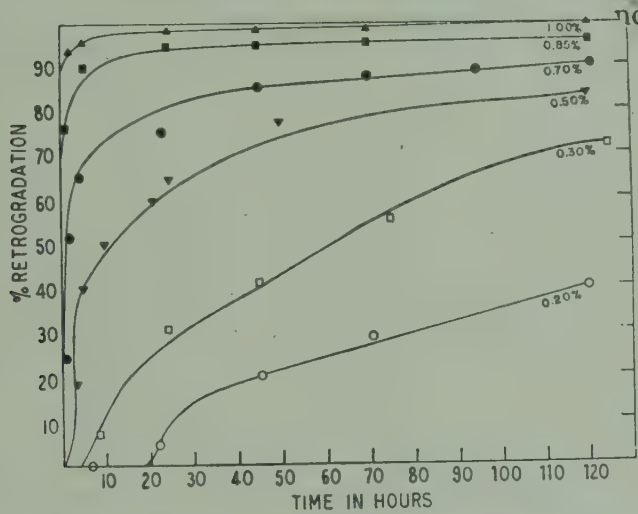

FIG. 8:3.—Effect of concentration on the rate of retrogradation of corn amylose in aqueous solution at 0° C.

Small variations in *p*H around the neutral point apparently do not affect the rate or extent of retrogradation. Weak acids such as carbonic, boric, silicotungstic or phosphotungstic are without action on retrogradation.[31] Low concentrations of sulphuric, hydrochloric, phosphoric or other strong acids, however, are said to increase the rate of retrogradation.[3, 9, 31, 32] Sulphuric acid, even in amounts up to 1 per cent. has been found[9] to increase the rate of retrogradation of potato starch. Smirnov[33] has reported that the highest rate of retrogradation occurs at *p*H 2, at which point the rate is approximately four times that at *p*H 6.

Since alkalis are known to promote the dissolution of starch, no

cur in solutions of high *pH* retrogradation would be expected to been borne out by experi- value. In general, this opinion A Maquenne[3] and Roux,[9] have ment.[3, 9, 31] None the less, h effect of large amounts of alkali. claimed that in contrast to d to increase retrogradation. These small quantities of alkali ncongruous with the known character- statements seem to beour. One explanation may be that since istics of starch behaRoux used potato starch, sufficient phosphoric both Maquenne andailable in the starch to neutralise small quanti- acid groups were prevent a large change in the *pH* value of the ties of alkali an hydroxides of barium, calcium and strontium solution. Th ported to cause precipitation of starch solutions.[18, 31] have been known whether these alkaline earth hydroxides simply It is no p retrogradation or whether they precipitate starch in the speed of a complex. form

Various inorganic salts and organic compounds, when present in small amounts, accelerate the retrogradation of starch solutions. It is well known that most water soluble substances when employed in high concentrations will rapidly ' salt out ' or precipitate starch from solution. This rapid heterogeneous precipitation of starch is not considered a retrogradation phenomenon. On the other hand, retrogradation of starch from solution is speeded by the presence of small amounts of lead acetate, barium acetate,[34] calcium chloride, calcium sulphate,[35] ferric sulphate [36] or sulphate in general.[37] The salts investigated by Samec and Benkovie[38] may be listed in the order of their increasing effectiveness in NH_4SCN, NH_4SCN, NH_4CI $(NH_4)_2$ SO_4. The effect of various accelerating retrogradation as follows: Na_2HPO_4, $Na_2B_4O_7$, salts appear to be in the same order as the Hofmeister lyotropic series. Retrogradation is also accelerated by tannin[39, 40] chloroform[40] and ether.[41] Certain proteins, amino acids and sugars have a mild retarding action on retrogradation. Surface active agents may alter the retrogradation rate and a few exhibit a pronounced retarding action. Some wetting agents of the anionic aryl-alkyl-sulphonate type strongly inhibit retrogradation.[19b]

Wolff and Fernbach[42–49] and others[4, 50] have claimed that grains contain an enzyme which, when added to starch pastes, increases the rate of deposition of insoluble material. This purported enzyme was named amylo-coagulase. The enzyme is said to accompany diastase generally. This would explain the increased rate of precipitate formation which is often observed to occur in starch solutions immediately following the addition of large amounts of diastase. Once the precipitation of starch is initiated, in this manner, heating of the solution to 120° C. to

destroy the activity of the diastase has no effect on stopping the deposition.[43] A malt extract heated to 120° C. before it is added to a starch paste is without effect on the rate of formation of the deposit. To produce the precipitation effect with malt diastase, it is said[44] to be necessary to obtain a good dispersion of the starch by heating the solution under pressure or by the addition of diastase free from amylo-coagulase. If to these prepared solutions are added an infusion of barley, rye, or wheat the deposition of insoluble matter is initiated at once.[45] Wolff and Fernbach[49] believe that conclusive proof for the existence of amylo-coagulase is shown by the fact that rabbits which have been injected with malt extract, develop in the blood serum a substance of anti-amylo-coagulase activity.

Existence of the enzyme amylo-coagulase is doubted by Sallinger[51] and Berczeller.[52] Sallinger has found that ptyalin causes precipitation in starch pastes. He has attributed this action to the hydrolytic removal of that part of the starch which served as a protective colloid, thus permitting the remainder of the starch particles to undergo rapid association. Berczeller believes that the enzyme merely decreases the paste viscosity, and by this action facilitates the aggregation and coalition of starch particles. In view of the known ease with which starch forms complexes with other compounds, it is possible to understand how large amounts of enzyme may simply precipitate a portion of the starch as a carbohydrate-protein complex. From these considerations it is evident that further work must be done before the existence of amylo-coagulase is generally accepted.

Changes Occurring during Retrogradation and the Properties of Retrograded Starch.—As a starch paste ages, the first indication of change is an increased resistance of the starch to hydrolysis by amylolytic enzymes. Autoclaved starch pastes allowed to stand for only an hour have a lower saccharification limit than fresh pastes.[2, 4] The retrograded starch precipitate is quite resistant to hydrolysis by amylases.[1, 4, 5, 8] Because the amylases are so sensitive toward retrogradation effects they provide an excellent tool for the detection of incipient retrogradation and for following the rate of retrogradation in the early stages. Sallinger[14] has used ptyalin to follow the rate of retrogradation of starch pastes.

Another change which can be noted early in an ageing starch solution is the increase in opacity of the solution. This manifestation of retrogradation is not so delicate or sensitive an indication of the ageing process as is the change in enzyme hydrolysis, but by observing opacity changes one can follow the progress of

retrogradation for a longer period. Ordinarily, the decrease in light transmission[26, 33] of the solution is measured in place of opacity or turbidity.

Starch solutions of low concentration (approximately 2·5 per cent. or less) decreased in viscosity on ageing.[13, 41, 53, 54] This decrease occurs due to removal of a part of the starch through continued precipitation of the aggregating particles. Solutions of higher concentration, on the other hand, set to a gel.

Samec has reported that as a starch solution ages an accompanying increase in conductivity occurs.[41, 53] This observation has not been fully explained. However, Samec worked with solutions of potato starch, which unlike some other starches, has phosphoric acid groups chemically bound to the amylopectin. It is possible that some of these groups were hydrolysed during the ageing process although this would not be expected under normal conditions.

As retrogradation proceeds towards completion a starch solution loses its ability to produce a blue colour on addition of iodine. Successive tests show that the colour changes from blue through purple or purple red to light pink. Maquenne[1] and others[68] have noted that iodine does not easily colour the deposit which settles out of an ageing solution.

In a concentrated starch paste or heavy gel, advanced ageing effects may be detected by X-ray photographs which show that the starch changes from an amorphous to a semi-crystalline condition. The intensity of the crystalline pattern increases as retrogradation progresses.[13] In every photograph, however, there exists a diffuse halo which indicates that a part of the starch remains in the amorphous condition. All precipitates from retrograding starch solutions appear to possess a partial crystallinity. As nearly as can be determined, starches of all types pass on retrogradation into the crystalline state,[67] typified by the 'B' X-ray pattern. This pattern is believed to represent starch molecules in an extended uncoiled form.[55]

Maquenne[1] has observed that retrograded starch is not easily hydrolysed by dilute mineral acids.

Retrograded starch is only slightly soluble in water at ordinary temperatures and redispersed with difficulty even at higher temperatures. Part of the retrograded material dissolves at 80-100° C. but autoclave treatment at 115-125° C. for about 30 minutes is necessary to bring about complete solution. Meyer, Bernfeld and Wolff[30] have reported that moist retrograded amylose will redisperse in warm water. If the retrograded starch is allowed to become dry, it is found to be much more difficult to redis-

solve and becomes soluble only when autoclaved[8] at 155° C. Moist retrograded starch, not previously allowed to dry, dissolves readily in sodium or potassium hydroxide[1] at room temperature, but dissolves much less easily if it has been previously air dried. The latter product, however, quickly dissolves in 1 N sodium or potassium hydroxide if the mixture is cooled[56] to 0-5° C.

Physical Nature of Retrogradation.—All evidence indicates that retrogradation is a purely physical phenomenon which arises from the tendency of starch molecules to associate. Ideally, a starch molecule in solution may be viewed as being highly solvated. In time, the kinetic energy of the solution brings together larger or smaller segments of two molecules, whereupon a part of the attached solvent is lost, and the segments are held by intermolecular forces. The length of intermolecularly-bonded segments may possibly grow as the remaining parts of the molecules move into proximity with each other. Once these retrograded nuclei are formed, they may serve as points for association or deposition of other molecules. Thus, colloidal particles are formed. In a starch solution aged for a short time, such colloidal particles, so small as to be undergoing Brownian movement, may be seen in the ultramicroscope.[57, 58] In time, as the particles grow, Brownian motion lessens and is lost.[58] Eventually the particles, either through continued deposition of molecules or by coalition with other particles,[71] become of sufficient size to precipitate.

This description of the retrogradative process accounts for the various changes observed in ageing starch solutions and explains many of the properties of retrograded starch. It explains the observed increase in turbidity of an ageing starch solution, as well as the crystallinity of the precipitated particles. It also explains why the retrograded molecules, because of their strong intermolecular forces, are not easily dispersed in water and are not available for attack by hydrolytic enzymes or for combination with iodine to give the familiar blue colour.

Amylose molecules, due to their linear nature, can associate easily and therefore retrograde rapidly. Amylose retrogrades so completely that ordinarily less than 0·04 per cent. remains in solution.[30] In addition amylose forms gels with surprising ease. A 5 per cent. solution of corn amylose allowed to cool to room temperature forms a rigid gel which is not dissolved by heating to 130° C.

Amyloses from different starches retrograde at different rates. A comparison of the rates for corn, wheat and potato amyloses is shown[19a] in Fig. 8 : 4. Potato amylose, although of a higher

molecular weight than corn and wheat amyloses, retrogrades more slowly than these. It has been suggested[59] that the slow retrogradation of potato amylose may be explained by assuming that the molecules are not linear but are V-shaped and hence do not

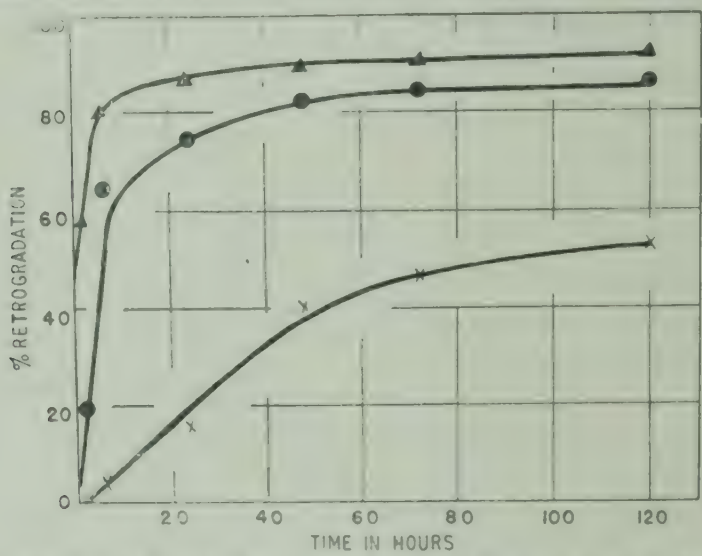

FIG. 8:4.—Rate and extent of retrogradation of amylose from 0·85 per cent. solutions at 25° C. ▲ wheat, ● corn, × potato.

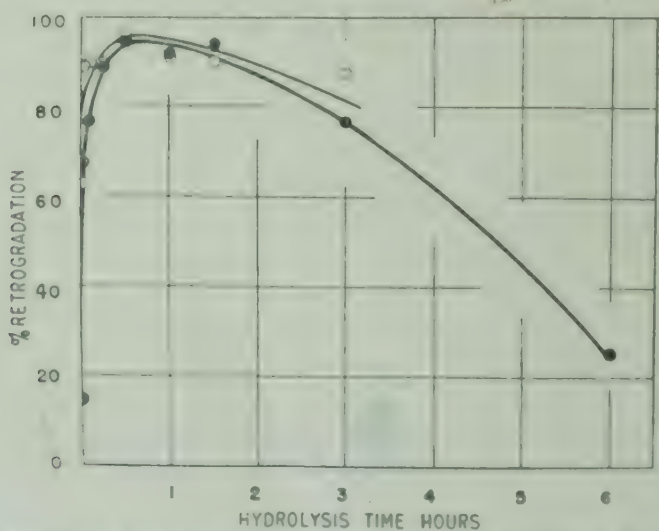

FIG. 8:5.—Effect of acid hydrolysis on the extent of retrogradation at 25° C. O corn, ● potato.

align with one another easily. Whistler and Johnson,[194] however, show that as the average molecular size of potato amylose molecules is reduced by mild acid hydrolysis, the extent of retrogradation during 24 hour period increases to a maximum and then decreases (Fig. 8 : 5). This suggests that for amylose molecules

there exists a critical size for which retrogradation rates are at a maximum. The maximum corresponds roughly to reduced viscosities of 0·45 to 0·56. Molecules of larger size retrograde more slowly, presumably because of steric effects resulting from their more coiled, kinked or convoluted structure. Molecules of smaller size retrograde more slowly, presumably because of the increased solubility inherent in a lower molecular weight molecule. Corn amylose, though lying nearer the maximum rate of retrogradation, goes through a similar increase and decrease in rate of retrogradation when its molecular magnitude is decreased by hydrolysis.

In contrast to amylose, amylopectin retrogrades very slowly.

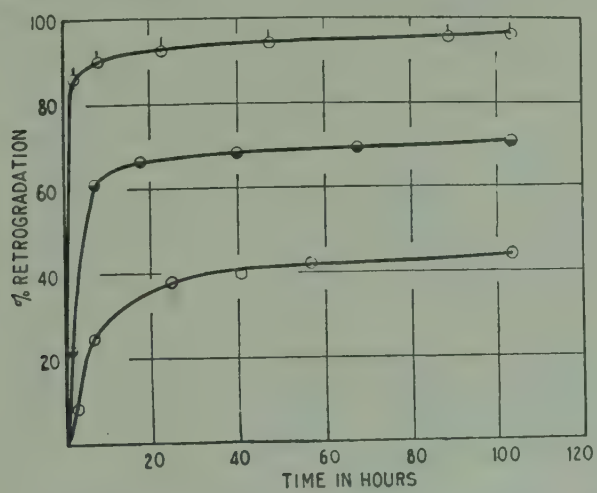

FIG. 8:6.—Rate of retrogradation of amylose-amylopectin mixtures at 0° and 0·85 per cent. concentration. ○ 90 per cent. amylose, ◒ 75 per cent. amylose, ○ 50 per cent. amylose.

Molecules of amylopectin, because of their branched nature, can associate only with difficulty. One of the reasons why waxy corn starch retrogrades so slowly (see Fig. 8 : 1) is that it is composed entirely of amylopectin.

In a normal starch dispersion the retrogradation of amylose is retarded by the amylopectin. The rate and extent of retrogradation of any particular amylose-amylopectin mixture varies in direct ratio with the amount of amylose present. This is more clearly demonstrated in Fig. 8 : 6 which shows the rate of retrogradation for several mixtures containing different ratios of amylose to amylopectin.[19b]

When an artificial mixture of amylose and amylopectin is allowed to retrograde the amylose tends to separate out first. Hence, such initial precipitates are relatively rich in amylose. Likewise, during the retrogradation of some dispersed whole

starches, such as tapioca, arrowroot and white potato, it is observed that an initial rapid precipitation of amylose occurs. This fractionation of the starch components is partly evident from an examination of the retrogradation rate curves shown in Fig. 8 : 1. Other starches do not exhibit an early preferential precipitation of amylose but deposit material with about the same composition as the original starch. Such behaviour may infer that the amylose and amylopectin are more closely associated in these starches, and that complete molecular dispersion does not easily occur.

In starch solution containing 3 per cent. to 5 per cent. or more of starch, the retrogradative effects do not result in a settling out of flocculent particles but in the formation of a gel. At these higher concentrations the solvated molecules easily touch and associate with each other at various points to form a typical open gel structure. On continued ageing, the molecules become linked over a greater part of their length, until eventually there develops a degree of crystallinity detectable by X-ray analysis. This association may be sometimes accompanied by an observable syneresis.

Retrogradation is prevented, usually by the presence of alkalis, amines, aldehydes, phenol, urea, acetamide, etc.[41, 60] These chemicals presumably bond to the starch and prevent the starch molecules from combining with one another. For a similar reason, water soluble derivatives of starch, such as the partially substituted esters or ethers, do not retrograde.

Significance of Retrogradation in Industry.—Retrogradative changes are of importance in the industrial use of starch pastes and in starchy food products which are maintained in a moist or wet condition.

Immense quantities of starch are used in the form of a solution or paste as textile sizes or finishes or as adhesives. For uniform operation on the machines and for even application of the starch, it is imperative that succeeding batches of solution or paste be identical with each other and maintain their initial properties throughout the time they are being used. The occurrence of viscosity changes or the formation of a precipitate in the starch mixture is detrimental. These retrogradative effects are avoided or curtailed by holding the solutions at high temperatures and by use of modified starches such as dextrinised or oxidised starch in which the possibility of retrogradation has been reduced. Even in some dextrins, however, retrogradative effects slowly occur as evidenced by the 'whitening-back' of the solution and by the decrease in gloss of envelope gums.

In canned foods such as corn, peas, soups, etc., retrogradative

effects may produce undesirable changes in texture. So far, however, little is known of the retrogradation process occurring in these products.

The staling of bread has been attributed to a retrogradation of the starch within the loaf. Staling is a rapid process ; at temperatures of 2-21° C. the greatest amount of staling occurs in the first 25 hours.[61–62] The obvious change in bread as it stales is that its texture changes from a soft to a hard, granular state. It loses its characteristic cohesiveness and becomes crumbly. Bread is a complex mixture of starch, protein and other constituents and possibly the staling process embodies interrelated changes in several of the bread components. Katz, however, after a series of intensive studies, has concluded that retrogradation of the starch is the prominent factor which brings about staling. Since bread may be considered as a highly concentrated paste a[illegible] rate of retrogradation may be expected. [illegible] M. HEDGES

Bread stales most rapidly[61, 62] at 2-3° C. but [illegible] at temperatures above 60° C. Fresh bread on ex[illegible] 15, 541. produces an amorphous or perhaps a ' V ' or pas[illegible] staling occurs the pattern changes to the retrograde[illegible] 182, 100. Stale bread exhibits a marked resistance to hydroly[illegible] Acta., 1940, 23, lytic enzymes.[64]

True staling is not a mere hardening or drying out [illegible] since bread stales [illegible] when it is placed in a sealed container. The particles of bread do become more compact, however. The staling process is accompanied by a decrease in the swelling power of the crumb and by a decrease in the amount of starch which can be extracted with water.[60] Katz[60, 65] and Verschaffelt have examined fresh and stale bread microscopically, and have found that in contrast to the condition in fresh bread, the starch granules in stale bread have pulled away from the protein matrix and are sharply outlined due to a very thin surrounding layer of air. Ostwald and others have suggested that staling may be due to a syneresis. Kuhlmann and Golosowa[66] followed the amount of ' bound water ' in bread throughout the process of baking, cooling and staling. The water-binding capacity decreases at the conclusion of the baking process, and becomes smaller during the period of cooling and subsequent staling.[66a]

Katz and Weidinger[22] have assumed that in the staling process the starch first separates into viscous droplets (coacervates) which then crystallise.

If staling is due to starch retrogradation, one might assume, at first, that the process results from changes in the amylose fraction. Several workers,[69, 69a] however, have recently supplied evidence to

indicate that the effects are due to retrogradative changes of the starch amylopectin. Schoch and French[69] have evidence which suggests that the amylose in bread is insolubilised by retrogradation during the baking and cooling of the loaf. This is based on the fact that soluble material leached from fresh bread is largely amylopectin. As staling progresses the amount of extractable amylopectin decreases, thus providing indication that the amylopectin is undergoing retrogradation.

Staling may be prevented or retarded by holding the bread at a temperature[63] of 60° C. or by rapidly cooling the bread and maintaining it at a temperature[60, 62] of −10° to −20° C. The various chemicals (pyridine, acetaldehyde, caustic soda, etc.) which normally inhibit retrogradation will also inhibit bread staling, but, the use of these substances in bread is impractical. It is inter- with ea to note that the sugar arabinose is said to inhibit the staling ture. On[70]
greater part or siderable importance has been given to the addition degree of crystal ce active agents to bread doughs. Some of these tion may be som rked anti-staling properties. Polyoxyethylene,

Retrogradatio and monoglycerides have been found effective as amines, aldeh ents when added to flour doughs[72–75] and they have chemicals pr ed in commercial baking (see Chapter 10, Vol. 2). molecules rface active agents have been found to be effective in reducing the rate of bread staling; th agents are glyceryl monostearate, glyceryl oleostearate, sorbitan monostearate, polyoxyethylene sorbitan monostearate and polyoxyethylene monostearate.[76] The latter substance is most effective. The action of this agent and of other ingredients of bread on the rate of firming in the bread crumb have been examined.[77] Polyoxyethylene monostearate increases the maximum viscosity of wheat flour pastes and retards the dextrinisation time with alpha-amylase.[78] The effect of the surface active agent seems to be one of reducing the hydrophilic characteristics of the starch so that the normal molecular alignments of starch chains with each other and with water fail to occur.[79] Various methods have been examined for measuring the changes which occur during the staling of bread.[80, 81]

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Chapter 9

STARCH AND THE HYDROGEN BOND

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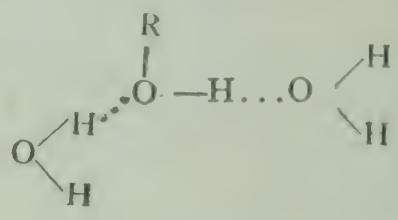
In recent years the conception of a new form of valence linkage through hydrogen (the ' hydrogen bond ') has been exhaustively investigated and experimentally established. Its literature is growing very rapidly, and the scope of this chapter does not permit of an adequate review. A few references only[1–8] must suffice. At present the nature of the bond is regarded as largely ionic, in effect a hydrogen bridge between two electronegative atoms, viz. O—H ··· O, N—H ··· O, N—H ··· N, N—H ···F, etc., using Pauling's designation (—H · · ·). The discovery of this form of secondary valence promises to be one of the most important in the history of physical chemistry, and can no longer be ignored in the study of many types of compounds. Hydroxylated compounds, especially, have been found to associate through this form of linkage, and expression has been given to the thought that it might play an important role in starch and cellulose structure and behaviour.[5, 6, 9–12]

When an aqueous dispersion of a starch is hydrated, let us say, by cooking, the initial physical reaction is a swelling or gelatinisation of the granular packages. This effect is largely a function of temperature of the water, although starch granules may become more or less swollen at room temperatures if allowed sufficient time. But temperature is the primary factor, and for a given botanical variety of starch there will exist a temperature range (usually narrow) within which the bulk of the granules swell or hydrate. This gelatinisation range may also be affected to some extent by the mode of manufacture of the starch. What is the very interesting mechanism of this hydration ? Why is hot water, above a certain temperature, effective, and cold water relatively ineffective ? And why do sufficiently high aqueous concentrations of certain salts, such as NaI, NaSCN, etc., gelatinise starch in the cold ?

The hydrogen bond suggests a very plausible and probable

answer to these problems. That hydration, in general, is some form of association with water molecules through hydrogen bonding is now accepted by many physical chemists. In the case of starch hydration, the phenomenon may be represented as follows :



water penetrating the highly organised granule, and swelling it as the water becomes available to the clustering hydroxyl groups. In order to be available to the starch hydroxyls, the penetrative power of the water must be high. Now water itself is known to be 4-co-ordinated, or associated,[13] its hydrogen bonds, in the liquid phase, being continuously destroyed and reformed, the destruction rate being highest at elevated temperatures—as would be anticipated from bond energy considerations. At lower temperatures in the liquid phase the relatively large size of the water molecular aggregates would impede penetration ; at higher temperatures these aggregates would be progressively dissociated until at some temperature dependent upon the closeness of the 'packing' of the particular starch aggregates, considerable penetration would ensue and the granules would swell or gelatinise.

This theory of starch hydration is supported by the well-known fact that starch is gelatinised in high aqueous concentrations of certain salts (see p. 87) and by the light that has very recently been thrown upon the effect of these salts upon the co-ordination of liquid water. Buswell, Gore, and Rodebush[14] have found through infra-red absorption studies that the 4-co-ordinated structure of water appeared to be at least in part destroyed by 4M concentrations of the lyotropic series NaI, NaSCN, NaBr, etc., the effect of NaI being > NaSCN > NaBr. Since NaI and NaSCN are effective at high aqueous concentrations in gelatinising starch in the cold, the above postulation of the mechanism of the hydrating action would appear to be singularly strengthened.

As gelatinisation proceeds—through cooking or other means—the larger swollen sacs rupture more or less and a heterogeneous jelly-like mass is formed, gradually becoming more dispersed and lower in viscosity or consistency. Dispersion is greatly promoted by mechanical agitation or by other physical means (see Vol. II) but becomes progressively more difficult owing to the elasticity

of the hydrated masses. Cooling, or even cessation of agitation, promotes an increase in consistency—in some instances, as in the cereal starches, amounting to a gel or a plastic paste.[15] These marked changes in consistency with change in temperature, or with quiescence or ageing, may be rationalised by the assumption, well warranted, of a network of hydrogen bonds acting through water aggregates and more or less immobilising them. If this network has a relatively fine structure, an irreversible gel may result; if relatively coarse, enough of the hydrogen bonds may be destroyed by reheating and agitation to permit of a renewed mobility of starch and water aggregates. By suitable physical processing the aqueous pastes of certain botanical varieties of starch may remain in a relatively mobile or fluid state over a wide temperature range and for long periods of time; other varieties will persist in reverting to a gel or to a plastic or semi-plastic state.

The extraordinary variations in the viscous properties of an aqueous starch paste are partly illustrated by the curves of Fig. 9: 1. Ten per cent. concentrations of a high grade tapioca or cassava were prepared by mild cooking, and two methods of physical dissociation (A and B). Method A involved thorough mechanical agitation by an ordinary stirring mechanism; method B involved special means of drastic physical disorganisation. The time for both treatments was approximately the same, also the temperature; and hydrolytic degeneration—as indicated by chemical tests*—was slight in both cases. Method B produced a clear fluid dispersion of exceptional stability and homogeneity. The paste produced by method A had to be diluted to 5 per cent. in order to permit kinematic viscosity measurements between 10° and 80° C. Curves 1 and 2 represent the plots of the log of kinematic viscosity (as measured by a 'modified' Ostwald pipette) against the reciprocal of the absolute temperature between the limits 10° and 80° C., curve 1 at 5 per cent. concentration, curve 2 at 10 per cent.

There is an astonishing difference between these curves, not only in their values but in their form. Being plotted on semi-log paper, the slope of the curves is representative of the percentage rate of change of viscosity as a function of 1/K. Curve 1 shows a relatively high percentage rate of change at both extremes of temperature; curve 2 is almost exponential, particularly at the higher temperatures. The latter is, in fact, remarkably similar to the form of the curve for pure water. It is obvious that curve 2 is characteristic of a fluid of a high degree of homogeneity, and that curve 1 is characteristic of considerable heterogeneity.

* Taylor's alkali labile method.

What is the cause of these profound differences in the viscous properties of the same starch ? Method B has not degenerated it into a thin-boiling type. The form of curve 2 is not that of a thin-boiling starch or 'white' dextrin, nor do chemical tests indicate a thin-boiling type. The only probable and satisfactory explanation appears to lie in phenomena of association through hydrogen bonding. The aqueous dispersion represented by curve 1 is a heterogeneous mass of micellar aggregates, many of

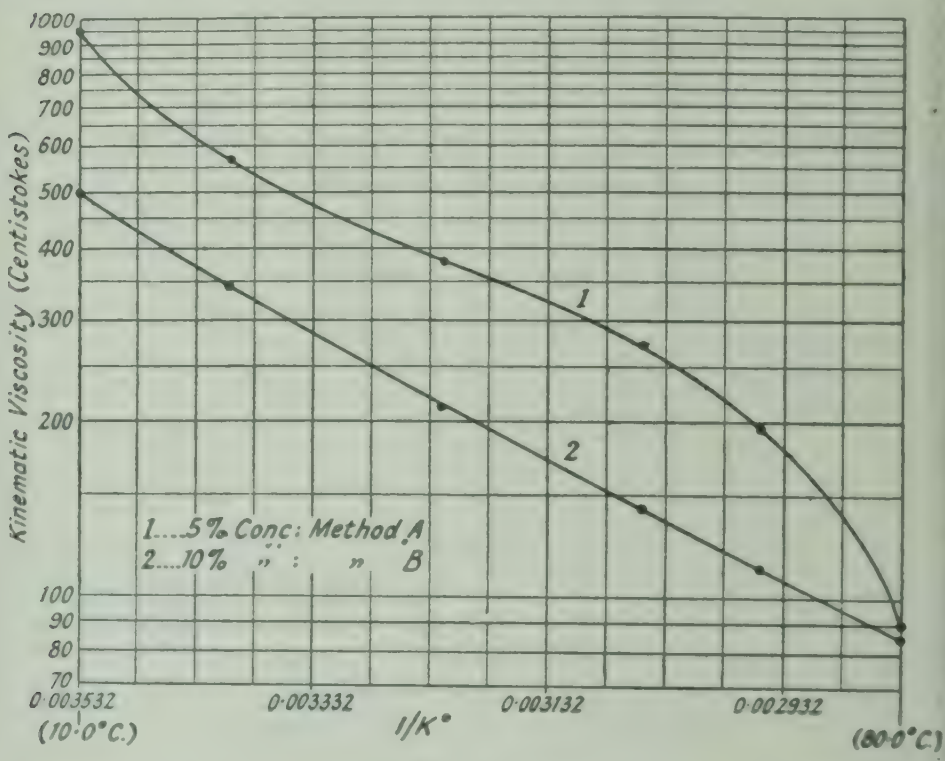


Fig. 9: 1.

them relatively large complexes of starch polymers associated through and with co-ordinated water molecules. We would expect the mobility of such complexes to be sensitive to temperature changes, especially at low and at high temperatures where the rates of formation and destruction, respectively, of hydrogen bonds would be greatest. The extended S-form of curve 1 is precisely what might be anticipated from these considerations.

But how about curve 2 ? Why is it so flat ; and considering that its solid concentration is double that of curve 1, why is it so extremely fluid, relatively ?

The profound disorganising treatment of method B has torn apart the clotted aggregates left by method A. The micellar

organisation has been sieved out,* as it were, into much smaller associated fragments, so small indeed that the water has in a very real sense become unbound, a result to which changes in the spatial configuration of the starch molecule itself may not too improbably contribute.[18] This is admittedly speculative. But in any event the form of curve 2 is, as has been stated above, remarkably similar to that of water or of other pure and more or less associated liquids. It might be added, parenthetically, that the plot of log of viscosity against the reciprocal of the absolute temperature is linear for an 'ideal' liquid. According to the theory of viscous flow postulated by Eyring,[16] the viscous behaviour of associated liquids must be abnormal and be expressed by a form of curve more or less similar to curve 2 . . . 'the energy of activation for viscous flow is not independent of temperature . . . and in addition to the normal work required to make a "hole", it is necessary for the hydrogen bonds to be broken before the activated state for flow can be attained'.[16] It is most interesting and suggestive that the same starch can be made to yield, through different physical processes, such a variation in viscous flow behaviour as shown by curves 1 and 2. Too few investigators of starch have had a thorough appreciation of the remarkable mutations to which it is subject in aqueous dispersion.

The case for the O—H . . . O linkage in starch is further supported by the viscous behaviour of 'chlorinated' starches. There is reason to believe that some of the secondary alcohol groups in the glucopyranose units are oxidised to ketose groups by hypochlorite treatment. Oxidation of this sort would obviously inhibit hydrogen bonding. So-called 'chlorinated' or oxidised starched might therefore be expected to show reduced associative tendencies, dependent upon the severity of hypochlorite treatment. This is confirmed by the viscous behaviour of aqueous dispersions of 'chlorinated' starches: they are phenomenally fluid, clear, and stable, as compared to the aqueous pastes from 'thin-boiling' starches modified by hydrolytic scission of main valence chains.

The case for the high-soluble 'canary' dextrins is similar. This type may be made very stable in viscosity, even at extremely high concentrations, exhibiting little tendency to associate upon ageing. A conception of the structure of these dextrins[17, 19] for which there is considerable support, postulates that they probably are, essentially, more or less etherised fragments of the original amylose. Here again, if this picture be approximately correct, association through hydrogen bonding should be reduced.

* Confirmed by the R.C.A. Electron Microscope.

On the other hand, a high-soluble ' white ' dextrin, manufactured with a higher acid content and at lower temperatures for shorter times, would be expected to contain a higher proportion of OH groups and to show different viscous properties. This is in agreement with the facts, the plot of viscosity versus temperature showing a characterically steep slope, merging at low temperatures into the plastic state, at high concentrations. This is strong presumptive evidence of the liberal presence of OH groups forming an O—H . . . O network.

In conclusion, the postulation of the hydrogen bond in starch and its derivatives makes for a much clearer understanding of their many puzzling anomalies of chemical and physical behaviour. The concept has, in recent years, gained a much wider acceptance.

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Chapter 10

THE REACTION OF STARCH WITH IODINE

The well-known action of iodine on starch to form the blue so-called 'starch iodide' has been extensively studied, and has given rise to much controversy since its discovery by G. de Claubry[1] in 1814; and the question does not seem to have been settled satisfactorily even yet. The fact that, as yet, we cannot explain fully the mechanism of the formation of the iodine coloration does not prevent its use as an empirical index of the degradation of starch. Degradation of starch by acid or enzymic hydrolysis, oxidising agents or thermal decomposition, produces a progressive change in the colour given with iodine and it is possible to separate dextrins of decreasing complexity which show colours varying from blue-violet, through violet and reddish-brown to pale red to colourless when iodine is added to them (see Vol. II). From a consideration of the colour developed by the α-amylodextrin, which is of the retrograding type, or of the 12-unit dextrin, which exemplifies the non-retrograding type, it does not appear that chain-length is the *sole factor* governing the particular colour with iodine.[74] At the same time there appears to be a lower limit to the chain-length below which no iodine coloration is produced. Dextrins of 6 units chain-length give no coloration[75] and those of 8 or 12 units a red coloration. At the point of disappearance of the iodine reaction, i.e. the achroic point, therefore, it is unlikely that any dextrins of chain-length longer than 6 to 7 units remain in the solution. The work of C. S. Hanes and M. Cattle[57, 59] is of importance (see p. 239).

G. de Claubry found that the addition of iodine solution to dilute solutions of starches obtained from many sources gave an intense blue coloration, and he also studied the effect of certain acids, alkalis and other reagents upon it. Leroy[2] found that water was necessary for the formation of the blue colour, an observation confirmed by H. B. Stocks,[3] who studied the action of iodine vapour on starch.

A number of workers[2–5, 8] have all suggested that the presence of hydriodic acid or an iodide in addition to water is essential for the development of the blue colour, and it has been suggested by

M. Samec that the iodide actually forms part of the complex formed, possibly as the polyiodide ion I'_3. Certain salts other than iodides also enable the reaction to take place, and intensify the coloration produced.[9]

Meineke[10] added aqueous iodine solution to a starch solution without any blue colour being developed, although the resultant solution was quite yellow; after adding a little potassium iodide, the blue colour appeared immediately. C. F. Roberts[4] added a chloroform solution of iodine, which had been carefully freed from hydriodic acid, to a starch solution and no blue colour was obtained; on treating this mixture in such a way as to produce hydriodic acid, e.g. by heating, or by adding acid or potassium iodide, the blue colour appeared. A. Vogel[11] records that iodine in absolute alcohol gives no reaction with dried starch.

The percentage of iodine found in the starch-iodide complex varies with the conditions of preparation, ranging from 3-23 per cent.[33, 76]

The Effect of Heat.—On heating to the boiling-point the blue solution obtained by the action of iodine on starch solution, the colour disappears, but returns on cooling, and, according to S. Pickering,[12] the temperature at which this colour disappears varies with the intensity it possessed before heating. A. Vogel[13] has also mentioned that higher temperatures are required to decolorise the solution as the concentration of the starch iodide is increased (see also addit. refs.), and this the author has confirmed. E. Sonstadt[21] heated strongly dried starch iodide to a red heat: it charred, and upon analysis one-fifth of the iodine was found in the residual carbon.

Sensitivity of the Reaction.—The starch-iodide reaction forms a valuable means of following the course of the conversion of starch to dextrins either by the torrification method or by means of the action of diastatic or hydrolytic agents; and its use in volumetric work is well known. As the degradation of the starch increases in the course of any process for producing dextrin, so the colour given by iodine solution with a sample withdrawn from the batch changes from a deep blue through violet and red to brown, and finally no colour at all is produced (Vol. II). The sensitivity of the reaction between starch and iodine has been studied by E. Chretien and H. Vandenberghe[22] in their work on another problem. They find that at room-temperature (19° C.) 0·15 ml. of N/100 I will give a distinct blue colour to a solution of as little as 1 pt. of starch in 50,000 pts. of distilled water containing 50 pts. of potassium iodide.

The first mention of strips impregnated with starch for use in

detecting iodine appears to have been made by L. Jonas,[23] whose strips were made of cotton. The sensitivity of the reaction has also been studied by a number of other workers.[10, 24–28] Two of these[27, 28] deduce that dextrins have a greater affinity for iodine than has starch, and that the intensity of the blue coloration obtained when soluble starch is used, is inversely proportional to the amount of dextrins present. The presence of non-electrolytes does not appreciably affect the sensitivity of the reaction,[29] although in presence of albumin an excess of iodine is required for the appearance of the blue colour.[30] The reaction is inhibited by the presence of chloral hydrate,[31] tannin, and certain phenolic compounds, such as pyrogallol and resorcinol, but not in the presence of phenol itself.[32] Electrolytes, such as acids and salts, tend to increase the sensitivity of the reaction,[9, 34] being maximum for potassium iodide solution,[10, 34] although, according to J. Pinnow,[26] sodium sulphate appears to act best. The presence of electrolytes also affects the colour under certain conditions[77] (*vide infra* Simerl and Browning). Excess of alkali inhibits the reaction,[35] and when testing prepared dextrins for matching it is necessary to ascertain if they have a strongly alkaline reaction; if they have, the dextrin solution must be neutralised with dilute sulphuric acid before carrying out the iodine test. The alkaline type of product is met with in the so-called ' arable gums ', which are dextrins containing a small percentage of soda ash and borax (see p. 161, Vol. 2). The precipitation of the starch-iodide complex brought about by the addition of salts can also be brought about if iodine and iodide are added in excess.[78, 79]

An Abnormal Starch-Iodide Reaction.—F. Dafert,[36, 37, 38] U. Kreusler,[37] and Y. Shimoyama[39] discovered that starch from the glutinous rice occurring in China gave a red or reddish-brown coloration with iodine, although aqueous extractions gave no indication that dextrin was present. Y. Tanaka[40] has studied this starch, and confirmed the findings of the earlier workers. In the last few years starches showing this abnormal reaction have received special attention, the reason for this behaviour, the absence of the straight-chain amylose from the granules, has been elucidated and is dealt with at length in Chapter 11.

The Composition of Starch Iodide from Whole Starch.—The exact composition of starch iodide has given rise to much speculation, and many workers considered that definite compounds of starch and iodine are formed.[41, 46, 47]

The fact that starch iodide can be precipitated in the presence of certain salts has been used as the basis of several methods for the estimation of starch (see Vol. II), compounds of constant

composition being obtained, providing the conditions of precipitation are rigidly standardised.

G. Rouvier,[42] suggests that the compounds $(C_6H_{10}O_5)_{16}I_{22}$, where n equals 2, 3, 4 or 5, are formed as the amount of iodine added to a fixed amount of starch is increased. These compounds, it is suggested, have the power of dissolving more iodine. The amount of iodine found in these compounds varies from 3 to 19·6 per cent. By dissolving starch in hot glycerine, and precipitating with a potassium-iodide—iodine solution, J. Toth[7] obtained starch iodides from wheat, rice and potato starches containing 22·6-22·8 per cent. of iodine. Using wheat or rice starch, the largest iodine content Rouvier could obtain was 19·6 per cent., and for potato starch the maximum was 18·6 per cent. In the presence of insufficient iodine a potato-starch derivative was obtained containing 13·5 per cent. iodine, whilst derivatives of wheat and rice starches contained about 9 per cent. It would appear that potato starch has the ability to bind more iodine than wheat starch under certain conditions.[47, 77, 82, 83] Under similar conditions, C. O. Harz[43] obtained figures of about 7 and 19 per cent. for the amount of iodine in the compounds formed in the presence of insufficient and excess iodine, respectively. M. Padoa and B. Savarè,[44] using a starch iodide containing about 19 per cent. iodine, carried out conductivity measurements, the results of which tend to show that starch iodide is an additive product of iodine.

All this work serves the useful purpose of stressing the variations in composition of the starch-iodine complex with variations in the procedure followed in its preparation. Thus any analytical method based on the starch-iodide reaction must be strictly standardised at every step and the procedure followed rigidly by other workers if their results are to have any validity or comparative value. Analytical methods based on the starch-iodide reaction are discussed on p. 241.

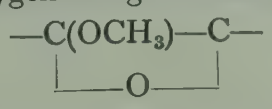
The Mechanism of Starch-Iodide Formation.—A number of workers[45, 49] hold that the formation of the starch-iodine complex is one of adsorption.[45, 69] Thus A. Lottermoser[48–49] obtained typical adsorption curves, his measurements being based on the determination of electric potential in the system free iodine/iodide ion as formulated in the Nernst equation, and also on the distribution ratio of free iodine between 0·1 N potassium iodide solution and carbon tetrachloride. L. Berczeller[50] found that no iodine was adsorbed by the starch from iodine solutions in carbon tetrachloride or benzene, whilst the amount adsorbed from alcohol was less than that from water. E. Angelescu and J. Mirescu[51]

have derived an exponential formula for the adsorption of iodine by maize starch. Conductivity measurements by N. R. Dhar[45] also point to the formation of an adsorption compound.

Other physico-chemical methods used in the study of the starch-iodine reaction include osmotic-pressure measurements,[52] measurement of the depression of freezing-point,[53] and colorimetric methods.[54]

M. Bergmann and S. Ludewig[55–56] suggested that the bridge oxygen atoms in starch are centres of absorption, and advanced this as a partial explanation of the progressive movement of colour towards the red end of the spectrum as starch is progressively degraded through dextrin to glucose.

It would appear that the iodine coloration is purely a question of adsorption depending ultimately on certain residual affinities of the starch molecule. Bergmann found that components containing the internal oxygen bridge of the type



such as the cyclo-acetals of acetoin and acetol, give blue-black compounds with iodine. Bluish compounds were also obtained by G. Barger and W. W. Starling[71] by the action of iodine on certain derivatives of α- and γ-pyrones, whilst colloidal or semi-colloidal substances such as saponarin and cholalic acid were found by Barger and E. Field[72] to give the 'iodine reaction' (see also Mylius[70]). W. Biltz[73] had previously observed the phenomenon with lanthanum subacetate.

From their work on amylases C. S. Hanes and M. Cattle[57] conclude that starch contains groups along the molecular chain which can adsorb iodine to give the characteristic coloration shown by starch and its various degradation products. As hydrolysis with β-amylase continues there is a progressive decrease in the number of adsorbing groups following the cleavage of successive maltose residues from the end of the chain. With α-amylase they consider that there is a preliminary disruption of the associated molecules, followed by a progressive destruction of the adsorbing groups as the chains are split into achro-dextrins. By means of their technique they find it possible to follow quantitatively the alteration in the iodine coloration under the action of different amylases, and so to delineate throughout the various degradation processes the relation between the iodine reaction and the reducing power. This provides a highly selective method for the recognition of points of similarity and difference in the action of various starch-splitting enzymes. They deduce that, in the visible region the

absorption spectrum of an aqueous solution of the iodine-iodide mixture is not appreciably altered by its participation in the starch-iodide complex.

Hanes's work led him to suggest[20] a helical starch structure with six glucose residues per turn in the helix. Dextrins with more than six glucose residues intensify the colour of iodine whilst those below this figure do not. A full turn of the helix, therefore, appears necessary for the formation of the starch-iodide complex.

Jackson and Hudson[80] have broken open the pyranose rings, giving aldehydic groups in positions 2 and 3 by oxidising starch with periodic acid. The product is highly colloidal but shows no iodine reaction, thus showing that the presence of pyranose rings is at least a partial condition for the starch-iodide reaction to take place. That the many OH groups present in the starch molecule play no part in the reaction is shown by the fact that fully methylated starch is still able to develop the blue colour.[79, 81]

Assuming boat-shaped glucose rings Freudenberg[91] built models of helical starch chains and found that such helices should have the hydroxyls directed outwards leaving a hydrocarbon lining to the centre of the helix. Since iodine solutions in hydrocarbons are blue, whilst water or other polar solvents give red solutions, he explained the blue starch-iodide colour by suggesting that the iodine entered the helix. It appears from further work that the above suggestions are correct, although it is clear that granular and retrograded starch do not possess a helical configuration. Gelatinised starch precipitated by alcohol possesses a crystalline structure which has been termed the 'V' modification by Katz, from Verkleisterung—gelatinised starch. Schoch,[93] and later Kerr and Severson,[94] produced crystalline amylose by the butanol precipitation method and in both cases an excellent 'V' type diffraction pattern was given by the product, the second preparation giving a pattern closely allied to the 'V' type even when still wet with butanol and water. Bear[95] drew attention to the fact that the X-ray diffraction pattern of starch-iodide was similar to the 'V' pattern. Dry granules or retrograded starch gives no iodine reaction with iodine vapour and the ability to absorb iodine vapour appears to be related to the quantity of 'V' pattern material present. Schoch and Kerr's materials are particularly good iodine absorbers.[96, 97] It would appear, therefore, that neither water nor iodide ions are essential for the formation of the starch-iodine complex as has been previously claimed but that their role is possibly confined to assisting in the formation of the 'V' configuration of the molecule. Dichroism of flow of starch-iodine complex in solution has already been discussed earlier,

and, with other optical properties, has provided one of the best checks on structure showing that the straight chain helical structure for amylose and the branched chain, nearly spherical shape, of amylopectin indicated by this and other work are not far short of the truth.

In another paper Rundle and French[98] give details of their study on the X-ray diffraction studies of the starch-iodine complex. The amylose-iodine complex has a hexagonal unit cell, $x = 12 \cdot 97$, $C_0 = 7 \cdot 91$, $d_{100} = 11 \cdot 23$ A.U. and the unit cell confirms a helical structure for the complex, 12·97 A.U. being the diameter of, and 7·91 A.U. the length of, a turn in the helix. These dimensions are in good agreement with the dimensions of a space-filling model of a helix with six glucose residues per turn. Although starch in the 'V' configuration will absorb iodine vapour in quantity, in the 'A' and 'B' configurations it will not. The maximum iodine absorption has not been established but amylose in the 'V' form will absorb 26 per cent. of its own weight of iodine.

R. H. Müller and M. H. McKenna[58] have examined the starch-iodide system by a photo-electrical method and find that beyond a preliminary 'dissociation stage' Beer's law is followed, and the use of Duboscq type of instrument in analysis is indicated.

Use of Starch Iodide.—Starch iodide is a strong disinfectant, and is comparatively stable. In contact with tissue it continues active for much longer than iodine itself, and if the iodine content is around 1 per cent. it is devoid of irritant action. The use of starch-iodide solution has also been suggested for the irrigation method of treating wounds.

The Starch-Iodide Reaction in the Spectro-photometric Determination of Starch.—Johnson[64] followed the course of enzymatic hydrolysis of starch by the iodine coloration and Blake[65] used the Duboscq colorimeter for this type of work. In many instances of routine testing there is no doubt that visual estimation and comparison of starch-iodide colours gives useful results but it is now generally agreed that the exact comparison of colours resulting from the addition of iodine to solutions of starches and starch fractions is only possible with the aid of the spectrophotometer or some similar device.[14, 57, 63, 87, 90] The degree of hydrolysis of modified starches has been followed by H. P. Das Gupta[66] and by G. B. Jambuserwala,[67] using the Lovibond Tintometer. Reference should also be made to the work of J. J. Chinoy.[68]

Several workers have made careful photometric studies of a few starches, and L. E. Simerl and B. L. Browning[63] have studied a number of starches and dextrins, using a General Electric Hardy

recording spectrophotometer, with special references to starches used in paper-making and the influence of extraneous material.

With an iodine concentration of 0·1 gm. per litre, and a potassium-iodide ratio of 1·5 to 1 by weight, the starch concentration does not affect the position of the minimum in the absorption curve ; neither small charges in the potassium iodide concentration

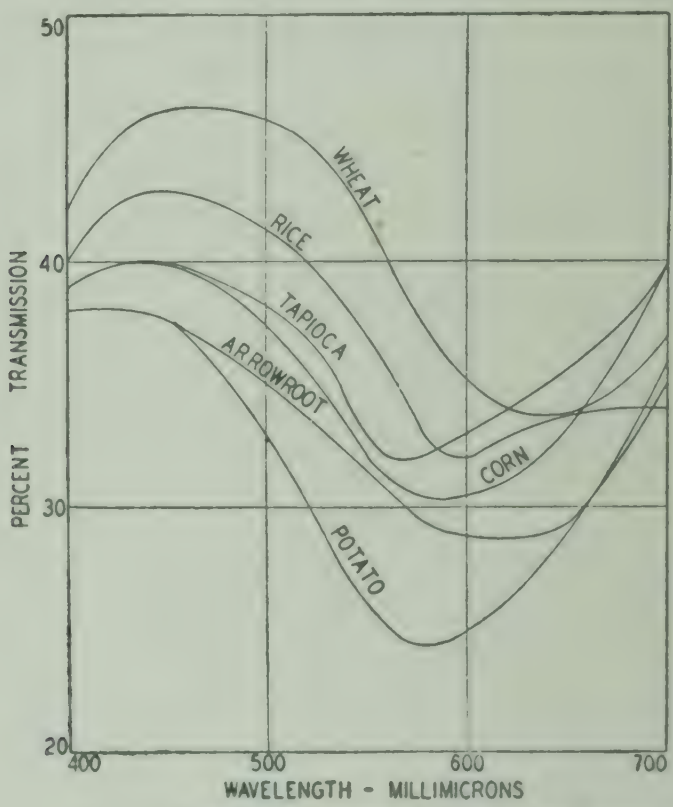


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FIG. 10: 1.

nor the presence of aluminium sulphate affect the minimum, although both affect the colour and the total absorption.

Fig. 10: 1 shows that the maximum absorption for the six starches examined occurs over the range of 5,600 to 640$\mu\mu$. It will be seen that the curves have the same general shape but considerable differences exist in the wave-length of maximum absorption and in the transmission at any given point in the spectral range.

Fig. 10 : 2 shows that an oxidised or hydrolysed starch has a different colour reaction with iodine, but that the transmission in the band of maximum absorption may not be uniformly effected. The maximum absorption, except for β-amylose and tapioca dextrin, occurs in the range 570 to 610$\mu\mu$. Modification to

dextrins eliminates the region of maximum absorption and the transmission rises rapidly and uniformly in the red.

At certain concentrations of iodine, deviation from Beer's law may be expected, but at higher concentrations, and by the use of suitable filters, the logarithm of the fractional transmission does become proportional to the starch concentration. In Figs. 10: 3-10 : 4 the transmission of the iodine solution with no starch present is assumed to be 100 per cent., and straight-line transmission curves are obtained over the range of 5 to 95 per cent. transmission. It will be seen that each starch has its own transmission curve having a slope which is characteristic of the starch type.

According to L. Paloheims and I. Antila,[61] the particle size of the starch influences the intensity of colour of the starch-iodine reaction, starches characterised by large particle size showing a lower transmission than those of small particle size.

Fig. 10 : 5 shows this relation under the conditions of Simerl and Browning's test,[63] but it should be noted that the relationship is inapplicable to modified starches.

Providing the kind of starch and its history are known, these workers consider that the photo-colorimetric procedure is capable of high accuracy, the nature of the starch itself being the limiting factor, and if raw starches are used, the deviation from the mean of the highest and lowest transmission curves of the starches examined amounts to ∓ 12 per cent. Fig. 10 : 5 illustrates that the particle size may be very important in determining differences in transmission of various starches, whilst for modified starches the possible error due to starch may be as much as ∓ 25 per cent. In the presence of dextrins, which may be present in paper together with raw or modified starch, the photo-colorimetric method becomes totally unreliable, whilst reducing agents and alkalis should be absent to obtain accurate results. Considerable errors may be introduced if the paper contains acid-soluble fillers and the starch is removed by acid extraction.

R. W. Kerr and D. R. Trubell[87] point out that the utility of starch as a size or adhesive depends on the characteristics of the component polysaccharides in the starch. The relative proportions of these components vary in various starches, particularly if they have been modified. They use spectrophotometric analysis of the iodine colours produced to determine the relative amounts of the two components present, as amylose stains blue and amylopectin reddish purple. They realise, however, the reservations that must be held against the method when used in evaluating various starch products for paper manufacture.

J. P. Nielson and P. C. Gleason[88–89] take into account the

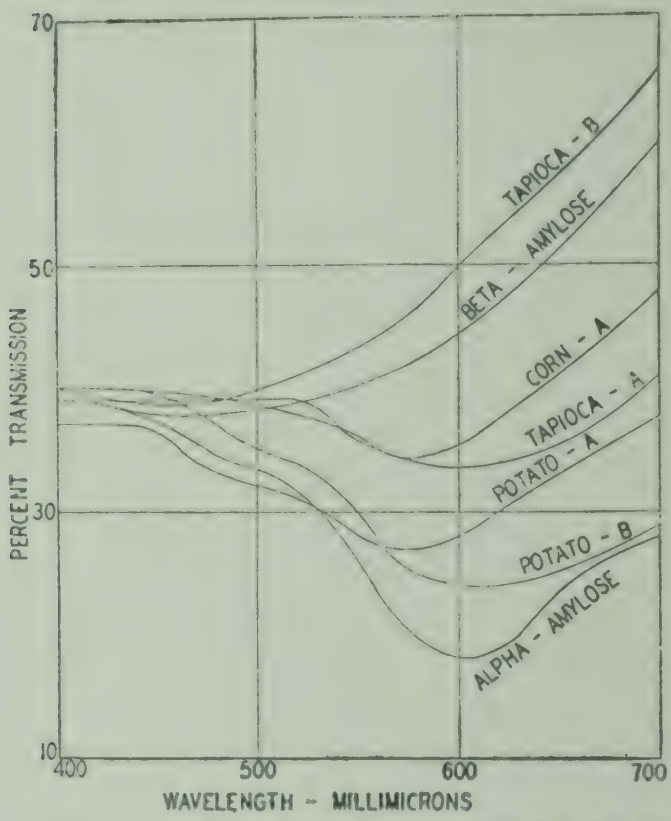

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FIG. 10:2.

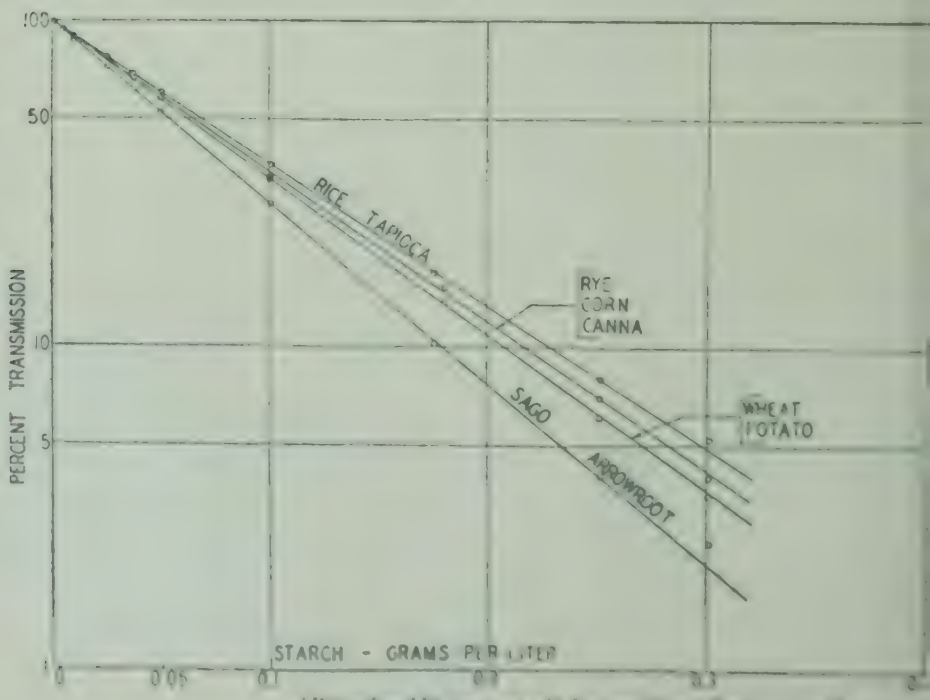

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FIG. 10:3.

different intensities of the starch-iodide colours produced by starches from different sources. They obtain the 'apparent starch' content by colorimetric means and then derive factors for converting these starch values into equivalent values for potato

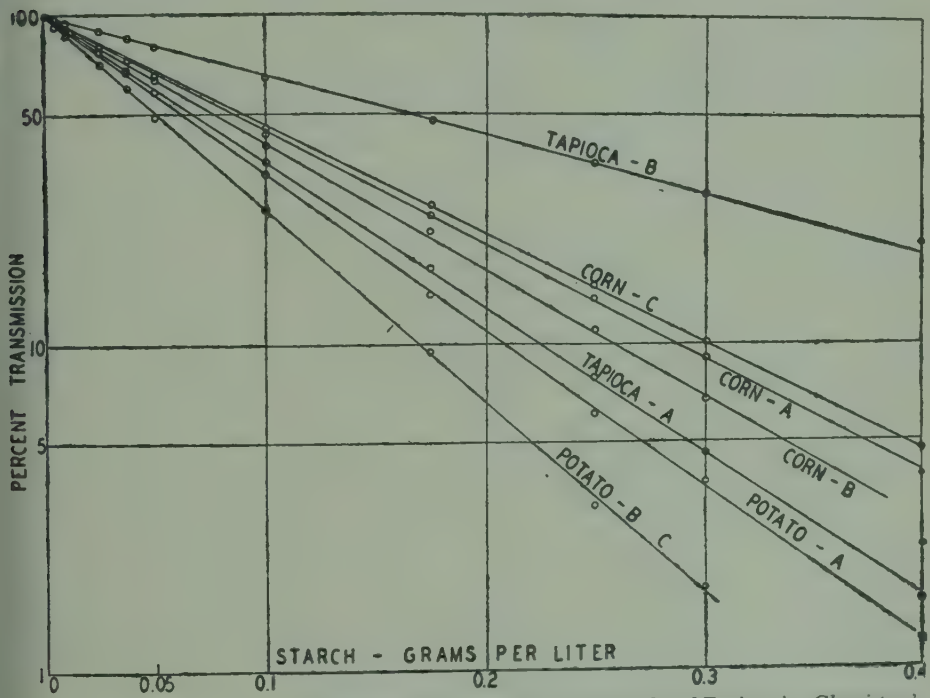

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FIG. 10:4.

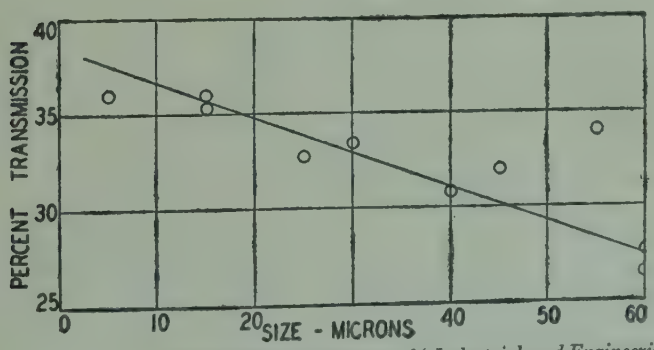

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FIG. 10:5.

starch. They obtain good agreement between the corrected values for starches from various sources by the colorimetric method and values determined by enzymic hydrolysis.

S. A. Watson and R. L. Whistler[15] point out that the iodine colour of some starches and starch fractions changes with the

concentration of the starch solutions and the amount of iodine added, therefore many of the starch-iodine colours recorded throughout the literature without an accompanying description of the test method, are not suitable for comparative work. They give a standardised method for preparing a fully-dispersed starch solution of known strength containing a known amount of electrolyte and at known *p*H and specify the amount of iodine solution (0·014 M KI) to be added. To facilitate and standardise the designation of the colour produced all colours are referred to standard colour charts[15, 16, 17] using the more precise notation of Munsell.[16]

A modified colorimetric starch-iodide method is described by H. Rath, D. Keppler and I. Roesling[19] for evaluating desizing materials. The rate of action, as distinct from the total activity, is indicated by comparing curves obtained by plotting the colour obtained under standard conditions, against the time to attain it for both the sample and equal weight of a standard enzyme preparation. A similar testing method using the actual sized fabric gives less reliable results.

Other applications of the starch-iodide reaction will be found throughout these pages. L. H. Lampitt, C. H. F. Fuller and N. Goldenberg[86] have examined the absorption spectra of the starch-iodide complexes formed from the different fractions obtained by ball-milling wheat starch (see p. 111).

They find that the absorption spectra given by the H.W.S. fractions (see p. 111) are unchanged by grinding except for a decrease in absorption at longer wave-lengths, which becomes apparent simultaneously with the first significant decrease in the mean molecular weight. The absorption by the C.W.S. fractions complexes are considerably lower than those given by the corresponding H.W.S. fractions, the greatest difference being in the visible part of the spectrum.

The C.W.S. fractions obtained by milling for 50 to 470 hours show a progressive increase in absorption but between 1,000 and 7,000 hours' milling the absorption, at the longer wave-lengths, decreases. When milled from 470-1,000 hours the two processes overlap. The mean molecular weight begins to decrease between 300 and 470 hours' grinding. These workers conclude that the depolymerisation of wheat starch brought about by 7,000 hours' milling is due to fission of the weaker, lateral links between the repeating units of 24-30 anhydroglucose units. The increasingly larger absorption of the C.W.S. fraction-iodine complex cannot at present be explained but it appears to be unconnected with the depolymerisation of this fraction by milling.

The change in molecular weight has a much less effect on the absorption at shorter wave-lengths of the starch-iodide complex, and changes in the starch are not easily followed by examination of this spectral region. The most sensitive region for the following of depolymerisation is from 4,300 to 7,500 Å, and the work of Lampitt *et al.* agrees very well with that of Hanes,[57, 59] Müller and McKenna[58] and Simerl and Browning.[63] Lampitt and co-workers found that increasing the ratio of iodine to starch causes the absorption of the starch-iodine spectrum to increase considerably, especially in the visible part of the spectrum, without appearing to reach a limiting figure, even after allowance has been made for the increased absorption of the iodine-iodide complex present.

Hence the absorption of the starch-iodide complexes appears to depend on both the relative and actual proportions of starch, iodine and iodide in solution. The characteristics of the curves given by the various fractions depend both on molecular weight and on the chain-length of the repeating units, the effect of the chain-length predominating.

It should be noted that several workers[82, 84, 85] have noticed that starch depolymerised to a considerable extent still gives the blue colour with iodine, but they did not examine the phenomena quantitatively.

Watson and Whistler[15] find the spectrophotometric method, carried out under the standardised conditions they lay down, to be particularly useful in determining the purity of amylopectin fractions. Quantities of amylose too small to be determined by the potentiometric titration method (see Vol. II) are readily detected in amylopectin fractions by this method and the addition of more iodine develops the true amylopectin colour. The persistence of the blue colour and its effect on the final colour gives an indication of the amount of amylose present. Larger quantities of amylose give such an intense blue colour that the development of the amylopectin-iodine colour is masked and prevents its detection. If more than 6-8 per cent. of amylose is present, therefore, the method does not allow the detection of the amylopectin present. Amylose-iodine complexes usually precipitate from such solutions. Under the conditions of the test employed by these workers waxy corn starch gives a true purple colour and not a red, as is generally reported. The red colour is only produced by the presence of excess iodine which contributes to the colour of the solution. M. M. MacMasters and G. E. Hilbert[18] have described the intermediate stages of colour that can be produced by the addition of increasing amounts of iodine solution to solutions of

waxy maize starch under identical test conditions, the various waxy starches seem to differ in colour as one proceeds from one type to another. Watson and Whislter list the starches in order of decreasing red and increasing violet content as follows: waxy rice, waxy corn, waxy barley, potato amylopectin and corn amylopectin.

This order coincides with the arrangement in order of chain-length of the respective molecules as suggested by Baldwin, Bear and Rundle.[90]

The oxidative effect of iodine on starch is fully discussed in Chapter 16.

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Chapter 11

THE WAXY CEREALS AND STARCHES WHICH STAIN RED WITH IODINE

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The not infrequent occurrence of starch granules which stain red with iodine rather than blue has been recognised for the past 60 years as having an important bearing on the structure of starches in general. Only recently has their relationship become clear from the standpoint of molecular composition. The blue-staining starches contain both a linear polymer of glucose (amylose) and a branched polymer (amylopectin) in varying proportions, but the amylose is entirely responsible for the blue iodine colour. The red-staining starches are composed almost entirely of amylopectin. Thus, their properties are similar to those of the amylopectin fraction from ordinary starch plus the characteristics imparted by their granular form. Our knowledge of the metabolic plant processes which determine the formation in some cases of red-staining starch and in others of the blue-staining form lags far behind our knowledge of their chemical composition.

Red-staining starches and the cereals containing them have alternately been termed 'glutinous' and 'waxy'. Both terms are equally misleading, giving rise to questions such as 'How much wax does waxy starch contain?' and publications entitled 'Do glutinous rices contain gluten?'[12]

Although waxy starch has recently been publicised as a new starch, the newness lies in the growing and milling of a waxy maize hybrid on a commercial scale and the introduction of the starch for industrial consumption. Red-staining starches were a subject of lively discussion in the botanical literature of 1887. The present review is intended to correlate information on the botany, genetics and chemistry of the waxy starches and the plants in which they occur. It is more complete with respect to maize than the other waxy cereals, since much of the literature on rice and sorghum has been published in unavailable Japanese journals.

I. The Waxy or Glutinous Cereals.—The widespread dis-

TABLE XII

Occurrence of Red-staining Starch

*Plant**	*Plant Part*	*Discover of Red-Staining Starch in Plant*
Greater celandine (*Chelidonium majus*)	Seed coat	Nägeli, 1858[87]
Rice (*Oryza glutinosa*)	Kernels	Gris, 1860[53]; Kreusler and Dafert, 1884[77]
Planetree maple (*Acer pseudoplatanus*)	Cotyledons	Sachs, 1862[87]
Orchid (*Goodyera*)	Ovary and embryo	von Treub, 1879[87]
Orchid (*Serapias*, or *Epipactis, lingua*)	Suspensors	von Treub, 1879[87]
Moth orchid (*Phalaenopsis*)	Ovary and embryo	von Treub, 1879[87]
Orchid (*Stanhopea oculata*)	Embryo and suspensors	von Treub, 1879[87]
Creeping rattlesnake plantain (*Goodyera discolor*)	Nearly all parts	Russow, 1884[87]
Orchid (*Goodyera repens*)	Nearly all parts	Russow, 1884[87]
Sheath addermouth orchid (*Malaxis*, or *Microstylis, monophyllos*)	All parts	Russow, 1884[87]
Orchid (*Epipogium*, or *Brassia, Gmelini*)	Rhizomes	Russow, 1884[87]
German Iris (*Iris germanica*)	Leaves	A. Meyer, 1882[87]
Yellow gentian (*Gentiana lutea*)	Near vascular bundles of leaf veins	A. Meyer, 1883[87]
Alpinebog swertia (*Swertia perennis*)		Russow, 1884[87]
Indianpipe (*Monotropa hypoptis*)		Russow, 1884[87]
Sorghum (*Sorghum vulgare*)	Kernels	A. Meyer, 1885[87]
Millet (*Panicum miliaceum*)	Kernels	Dafert, 1885[43]
Mace (*Myristica fragrans Hott*)	Seed coat	Tschirch, 1888[132]
Red algae (*Florideae*)		Bruns, 1894[78]
Canna (*Canna*)	Embryo	Overhagen, 1888[90]
Atlantic isopyrum (*Isopyrum biternatum*)	Tubers	MacDougal, 1896[82]
Garden onion (*Allium cepa*)	Root cap	Husek, 1902[90]
(*Alocasia putzeysi*)		Reichert, 1913[105]
Job's tears (*Coix lachryma-jobi*)	Kernels and pollen	Kempton, 1921[66]
Rice (*Oryza glutinosa*)	Pollen	Parnell, 1921[103]
Maize (*Zea mays*)	Kernels	Weatherwax, 1922[133]
	Pollen	Demerec, 1924[46]
	Kernels†	Andres, 1938[3]
Barley (*Hordeum sativum*)	Kernels and pollen	Kasiwaya, 1930[63]

tribution of red-staining starches shows them to be frequent products of carbohydrate metabolism in plants. A. Meyer in 1886[87], and again in 1920[90], summarised the sources where it was known to occur and these are listed in Table XII along with

* In cases where the original article did not give the common name, it was obtained from Kelsey and Dayton, *Standardised Plant Names*, 2nd Ed., 1942. J. H. McFarland Co., Harrisburg, Pa.

† A different waxy gene, wx_a, is responsible in this case.

those discovered later. But of those listed only the cereals such as maize, sorghum, rice and barley contain the starch in amounts sufficient for practical use.

Long before the difference in iodine colour was noted, the inhabitants of areas where waxy cereals were grown knew that they behaved differently from ordinary varieties when cooked. Thus glutinous rice was chiefly used in India and China as an adhesive or agglutinant, and the poorer people used it as food.[77] In China to-day, according to Chao,[30] it is universally incorporated into pastries for several national celebrations. It has long been utilised in Japan to prepare ' mochi ', a kind of cake ;[131] ' ame ', a sweet preparation by saccharification with malt ; and a liquor by mixing steamed glutinous rice, koji and spirits.[130] The straw of glutinous varieties of rice is more pliable and has greater tensile strength than that of starchy strains, both the grain and the straw commanding a premium on the market.[40] Red-staining rice varieties were known in China as Kiang-Mi, in Japan as Mochigome, and in Java as Kataa or Ketan.[87]

The properties of red-staining starch in glutinous sorghum made its meal especially suited for many cooking and technical purposes which differentiated it from ordinary varieties.[87] Glutinous sorghum has perhaps been cultivated in China since 2,000 B.C. under the name of ' Shu ', according to Collier.[31] He quotes Dr. Bretschneider, an authority on Chinese botany, as follows : 'This cereal is separately described in the Chinese Herbal published A.D. 1570. The grain is called Hwang-mi and is said to possess much glutinous matter. It is used for manufacturing alcoholic drinks.'

The discoverer of waxy maize[32] remarked that it was more glutinous than other varieties and might find use as porridge. In China, it was reported to be of limited distribution and did not make as good ' grits ' as other types ; however, its chief use in Upper Burma was not for the grain but for the husks which were used as cigar wrappers by the natives. Other varieties of maize had too coarse a husk for this purpose.[36] Mangelsdorf[85] attributed the distribution of waxy maize to natural selection rather than to intentional cultivation, pointing out that it is generally regarded as inferior to starchy types for food purposes. Waxy coix, a relative of maize, was used both for beads and as a cereal.[66]

The distribution of waxy cereals has already been touched upon —rice from Siam, India,[77] China, Japan and Java ;[87, 130] millet from China ;[43] sorghum from Japan and China ;[87] Andropogon sorghum from the Philippines ;[68] and coix, of which 14 waxy samples were discovered in 50 collected from Burma, China

India and the Philippines.[66] Of the 974 classified Philippine varieties of rice, 144 are glutinous ; and of 756 Javanese varieties, 41 are glutinous. About 8 per cent. of the rice area in Japan and as high as 20-30 per cent. of that in China is estimated to be planted in glutinous rice.[40]

Maize appears to have been the most extensively studied although among the last of the waxy cereals to be discovered. Reporting its first discovery in the vicinity of Shanghai, Collins[32] pointed out the possibility that waxy maize may have originated in China, although common maize originated in America. Following this discovery, 1,176 varieties of maize collected from all over the world (for another purpose) were examined but no waxy types found. A special study of waxy maize in China in 1915 revealed that it was of very limited distribution. The same year, however, 46 samples of maize from the Shan States in Upper Burma were found to include 19 with waxy endosperm. This region was relatively inaccessible because of mountainous terrain inhabited by head-hunters. Another occurrence of waxy maize was reported in Mindanao, Philippine Islands. Collins[36] suggested from this information that maize may have entered China from Burma. The first waxy maize found growing in America which had not been brought there intentionally was found in Connecticut.[84, 85] It occurred in an established variety of Sanford White self-pollinated ears and no other waxy varieties were known to be near. Thus it was believed to be a mutation from the common variety. Again in 1926, Bregger[15] found waxy maize growing in a vicinity where it had not knowingly been introduced, this time in the Argentine Chaco in an orange-yellow flint variety, Cuarenton. He raised the question as to whether it arose by mutation or came to South America from China.

During the years 1936-44, three mutations to the waxy character were reported to have occurred in inbred lines of corn among 100,000 selfs on one farm in Illinois.[8] These plants did not differ otherwise from the other inbreds and no other waxy lines were known in the vicinity. However, the mutation rate for the waxy gene (Wx→wx) is apparently very low. Stadler[122] determined the mutation frequency for eight different maize genes and found mutations occurring in all except the waxy, for which two million gametes were examined without occurrence of a single mutation.

Enomoto[50] selfed a glutinous variety of rice and obtained several heterozygous non-glutinous kernels. He attributed their occurrence to a gene mutation from glutinous to non-glutinous (gl→Gl), but the abstract does not mention whether he eliminated

other possibilities such as contamination by ordinary rice pollen.

Waxy maize from all the above-named sources proved to be genetically identical in that the waxy characteristic was determined by the same gene, *wx*, in homozygous recessive form. A genetically different type of waxy character, known as wx_a, has been found in Argentine maize[3, 4] and in barley.[63] Andres in 1938[4] detected two mutations to the wx_a character in lines of maize known as Colorado and Amarillo Fortin Refugio. Although the kernels did not differ from known waxy types in opacity, they were readily distinguished by their bluish-red iodine colour which was intermediate between the reddish colour of Chinese waxy and the blue colour given by ordinary non-waxy maize.

Notwithstanding the earlier literature relating the glutinous character to the red-staining property of starch from rice, sorghum and millet, fourteen years elapsed between the discovery of waxy maize (1908) and the report that it contained red-staining starch (1922).[133] During this time the genetic work on inheritance of the waxy character in maize was carried out using only the opaque appearance and waxy texture of the endosperm as a criterion. For dent corn* this is a reliable index and is used to-day to separate waxy from non-waxy kernels. An apparatus recently constructed for measuring differences in transparency of corn kernels by means of a photoelectric cell gives readings of 50-75 microamps for the relatively opaque waxy kernels as compared to 150 microamps for non-waxy kernels.

However, the kernels must be dried below a certain moisture content before this difference becomes evident. Andres[4] pointed out that in damp years the corn growing in the field may never become sufficiently dry to exhibit the opaque character and that under these conditions only the iodine-staining reaction can be used to differentiate waxy from non-waxy corn. The opaque appearance set in at about 13 per cent. moisture content. Temperature changes had no effect on this transformation except as they changed the rate of drying. The change from transparent to opaque occurred in portions of the endosperm separated from the rest of the kernel on drying, showing that the change was not controlled by the embryo.

The term 'waxy' or 'cerous' had not been associated with glutinous, red-staining cereals until Collins[34] so named 'waxy' corn because the texture of the cut endosperm resembled a hard, crystalline, opaque wax. The opaque,[130] dull white,[87] dead white,[103] greasy,[77] chalky[13] appearance of glutinous rice grains in

* The word *corn* as used in this article is synonymous with *maize*.

contrast to the glassy, translucent common variety has frequently been mentioned. For barley, however, only the iodine test serves to differentiate waxy and non-waxy varieties.[63] This is also true for sweet corn and floury corn where differences in opacity between waxy and non-waxy kernels are not evident.[67]

Parnell[103] was first to observe that the pollen of glutinous rice stained red with iodine. Soon afterward the same property was demonstrated in the pollen of waxy corn,[22, 24, 25, 46, 74, 75] waxy coix[81] and waxy barley.[63] A fact of great importance in breeding work is that plants heterozygous for the waxy character (Wxwx) bear equal numbers of red- and blue-staining pollen grains (see Fig. 11 : 2) be distinguished in appearance from non-waxy plants (WxWx), whose pollen grains all stain blue. By this means the waxy character can be carried along in heterozygous condition through successive generations of breeding and selection without the necessity of segregating it in pure form after each step. Longley[81] was able to judge the success of crosses between maize, euchlena and coix by using one waxy parent and observing the ratio of red- to blue-staining pollen borne by the hybrid.

The term ' red-staining ' actually includes starches which stain yellow, brown, red, purple and intermediate shades depending on the condition of the starch and the iodine solution used. Under the same conditions of staining, however, a sharp contrast is provided to the blue or blue-black colour imparted by ordinary starch grains. In order to be classified as waxy, a starch should contain little or no amylose and this can be determined by methods such as the potentiometric iodine titration.[7] To what extent the effect of foreign materials has caused amylose-containing starches to be reported as red-staining is not known. For example, small amounts of fatty acids can be bound by the amylose component of starch in such a manner that the formation of the blue complex with iodine is prevented.[108] However, in tissues containing a high percentage of oil, as in maize embryo, the starch stains a deep blue, apparently being uncontaminated by the oil. The early observations with respect to iodine colour of the waxy cereals have been repeatedly confirmed, but no further investigations seem to have been carried out on the red-staining starches of plants such as orchid rhizomes where experimental difficulties are greater.

Plants seldom contain waxy starch to the exclusion of the blue-staining variety. In waxy cereals the two types are restricted to certain tissues—the endosperm, pollen, and embryo sac[21] contain red-staining starch, while that of the embryo, seed coat, and the other parts of the plant is blue-staining. A. Meyer[87] presented a

coloured drawing of the cross-section of a glutinous sorghum kernel illustrating this condition. In iris leaves, red- and blue-staining granules occur side by side in the same cells. In the orchid, *Epipogium Gmelini*, the outer layers of young rhizomes are said to contain starch which stains copper-brown, while that in the inner parts stains blue. The oldest rhizomes contain red-staining starch throughout. Meyer further describes red-staining granules which stain blue after maturing (e.g. in *Dioscorea villosa* and *Aristolochia clematitis*) and granules from glutinous sorghum which stain with a blue-violet central portion surrounded by a red-brown peripheral region.

In order to explain the occurrence of granules giving all degrees of colour with iodine, Meyer proposed a hypothesis for their formation in harmony with the data available at that time (1886). According to his theory, starch was continually being deposited in the plant and digested again by enzymes so that there was a dynamic equilibrium between the insoluble blue-staining starch, the red-staining dextrins, and the soluble lower carbohydrates. The final proportion of red- and blue-staining materials in the granule was believed to depend on the rate of formation of the starch products, the ease with which the starch cleavage products diffused in and out of the leucoplast, and the activity of the enzyme. Since all three factors had to be properly balanced to get pure red- or blue-staining starch, most granules would stain somewhere in between these extremes. The weakness of this theory lay in the assumption that the red-staining carbohydrate was a less-organised degradation product of blue-staining starch, i.e. a dextrin. Samec[106] distinguished between red-staining starch (erythroamylose), and red-staining dextrins (erythrodextrins) formed by degradation of ordinary starch.

There is a question as to whether the differences in colour and intensity of staining within a particular granule correspond to an actual lack of uniformity in distribution of the granule components or are due to factors such as inability of the iodine solution to penetrate equally into all parts.[83] Sass[113] found that starch from Argentine waxy corn, which under certain conditions exhibits a gamut of colours with aqueous iodine solution, can be stained a uniform red-violet colour when a clearing agent such as lacto-phenol is employed.

The waxy gene (designated as *wx*) has been of great importance to the genetics of corn because its presence is readily detected and because it is inherited in simple Mendelian fashion, with slight deviations to be discussed later. Collins and Kempton[32, 34, 37, 38, 65] crossed waxy corn from China with common American varieties

having horny or starchy endosperm and found that the waxy character was completely recessive, no intermediate types being found. The kernels resulting from the cross were all horny, and segregated in the F_2 generation to give approximately a 3 : 1 ratio of horny to waxy seeds. They further crossed waxy corn (wxwxSuSu) with sweet corn (WxWxsusu) and obtained kernels of common corn, neither sweet nor waxy (WxwxSusu), which segregated out in the F_2 generation according to the dihybrid ratio 9 horny : 4 sweet : 3 waxy. This was later shown to be a 9 : 3 : 3 : 1 ratio when the iodine-staining technique made it possible to distinguish between waxy sweet and non-waxy sweet kernels.

Two other important results of their work were (*a*) the first demonstration of linkage in corn; and (*b*) the discovery that there were slight but significant deviations from the predicted Mendelian ratios of waxy to non-waxy plants.

(*a*) *Linkage.*—When Collins and Kempton[37] crossed waxy corn having colourless aleurone with non-waxy corn having red aleurone, the resulting kernels were non-waxy with red aleurone. However, in the F_2 generation, colourless aleurone was associated with waxy endosperm to a much larger extent than would be expected for independent segregation of the two characters. Conversely, if red-aleurone waxy were crossed with colourless-aleurone non-waxy corn, red aleurone remained associated with waxy endosperm in the F_2 generation. They extended this data[33, 65] and used it effectively to dispute the then current hypothesis that association between characters was determined by attraction or repulsion of the genetic units in a definite mathematical series. Their results provided evidence for Morgan's hypothesis that associated factors are inherited together on the same chromosome (linked).

Bregger[14] studied five of the factors necessary for development of red aleurone colour (*A*, *C*, *R*, *pr*, *i*) and determined that factor *C* was the one which Collins had found to be linked with waxy endosperm. He obtained cross-over percentages between *wx* and *c* of 26·7 per cent. as compared to 22 per cent. from Collins' data and 25·2 per cent. from those of Hutchison.[61,62] The latter investigator showed that the factors for shrunken endosperm (*sh*) and inhibition of aleurone colour (*I*) belonged to this same linkage group (later designated as Chromosome No. 9) in the order I-C-Sh-Wx. Since cross-over percentages are used to determine the relative positions of genes on the chromosome, the cause of variability in these values has received considerable attention.[39] The rate of crossing over between *c* and *wx* was found to differ

from one progeny to the next (even inherited in some cases), with sex (see also 120), with position of ear on the plant and with position of kernels on the ear. It was not affected by vitality of pollen, length of silk, weather conditions during anthesis, or time of shedding pollen.

The genetics of the waxy gene in maize has been investigated more extensively than in any other plant, partly due to the relative ease with which maize can be artificially pollinated. In contrast, the other waxy cereals are naturally self-pollinated and each flower must be tediously opened by hand when cross-pollination is required. Chao,[29] however, carried out linkage studies on rice and found the glutinous gene (*gl*) to occur on the same chromosome with genes for apiculus colour (ap4), leaf sheath colour (l_{sl}), tawny awn (t_y) and possibly stigma colour (s_{al}).

Emerson[49] has used the linkage between aleurone colour and waxy endosperm in the study of endosperm mosaics. When ears of waxy, white-aleurone corn (wxwxcc) were pollinated with pollen (WxC) from red-aleurone, non-waxy corn, most of the kernels resembled the pollen parent (red, non-waxy) but in a few cases there were found kernels containing small patches of white aleurone underlaid by waxy endosperm. He proposed that each of these areas may have developed from a single cell in which the chromosome bearing the two dominant genes (*Wx* and *C*) had been lost, perhaps by non-disjunction during cell division. Since endosperm tissue is triploid, the genotype of the aberrant cells could be represented as *wxwxcc*, while that of the normal cells in the endosperm would be *WxwxwxCcc*. Stadler[121] was able to increase the frequency of these endosperm mosaics from 0·08 per cent. to 2·56 per cent. by irradiation of the recessive ear with X-rays at pollination, and supported the view that all or part of a chromosome had been lost in these cells. The use of ultraviolet light instead of X-rays[123] usually produced the deficiency in the entire kernel rather than in portions of a kernel. Losses in red colour and non-waxy character usually occurred together, indicating that the change was due to loss of a chromosome section rather than a mutation from non-waxy to waxy.

In waxy cereals the cells containing one or three sets of chromosomes (pollen and endosperm) produce waxy starch, while cells with two sets (embryo) contain only blue-staining starch. The failure of expression of the waxy gene in the embryo is probably not entirely a consequence of its diploid condition, since red-staining starch has been reported in tissues of many plants which are diploid—in embryo, leaves, rhizomes, seed coat, etc. (Table XII). Further, if we accept Emerson's above-mentioned theory of

endosperm mosaics, the waxy character is expressed in endosperm cells with only two waxy genes. Brink[21] believed that the effect of the waxy gene is restricted to reserve starch, but this is not consistent with the evidence in Table XII.

(*b*) *Deviation from expected ratios.*—Collins and Kempton[37] found 23·1 per cent. instead of the expected 25 per cent. of waxy seeds in the F_2 generation of a cross between waxy and non-waxy corn. This was over nine times the probable error. Later Kempton[65] found a deficiency of 1·1 per cent. from the theoretical number of waxy kernels in a total of 102,427 seeds examined, which was too large to be due to chance. He suggested three possibilities to account for the deficiency—a higher death rate for waxy gametes, a less vigorous growth of waxy pollen tubes, or failure of plants to form waxy and non-waxy gametes in equal numbers. Brink[17, 18, 20, 21, 24] showed that this could not be attributed to a deficiency of waxy male gametes since an equal number of waxy and non-waxy pollen grains were formed in the anthers of heterozygous plants (see also 46); it was not due to a constantly different growth rate of waxy and non-waxy pollen tubes because kernel ratios on the upper and lower halves of each ear were the same. Chemical analyses of the two types of pollen showed no differences which could explain it. However, presence of the gene *su* for sugary endosperm increased the deficiency of waxy kernels—plants of the genotype susuWxwx when selfed gave 18·8 per cent. instead of the expected 25 per cent. of waxy kernels. It was further found that amylase from waxy pollen digested potato starch paste to less extent than amylase from non-waxy pollen. Brink suggested that the lower activity of amylase from waxy pollen in penetrating the silk coupled with slower pollen tube growth might explain the aberrant ratios. The lower growth rate was believed to be restricted to the first stage of pollen tube growth when it is dependent on reserves stored in the pollen grain.

Kiesselbach and Petersen[75] combined data previous to 1926 with their own which showed a deficiency of 0·7 per cent. waxy kernels from the 1 : 1 ratio expected in backcrosses and of 1·1 per cent. from the 3 : 1 ratio for selfs. They concluded that the most plausible causes were unequal fertilization due to differential pollen tube growth and systematic errors in pollination or kernel classification. Kempton[68, 69] failed to confirm many of Brink's results. He found that the ratio of waxy to non-waxy gametes was not consistently less in male than in female gametes. Differences in sexes were found, but in some cases the male and in others the female had a higher proportion of waxy gametes. The

percentage of waxy seeds on upper and lower ears in backcrosses was the same if the male were heterozygous, but if the female were heterozygous there was a larger percentage of waxy seeds on the upper ears of the plant. Long styles were not necessarily associated with a low percentage of waxy seeds. Gametes bearing both *su* and *wx* genes seemed to function as perfectly as those bearing other combinations of these genes. No differences in size, weight or specific gravity between waxy and non-waxy pollen grains were found. Kempton further made the important observation that the proportion of the two types of gametes functioning in fertilisation could be altered by external agencies. In backcrosses of waxy plants with pollen from heterozygous plants, the use of fresh pollen resulted in a deficiency of waxy kernels from the expected 1 : 1 ratio, but if the pollen were stored in daylight for about 7 hours, the waxy kernels were formed in excess of 50 per cent. Although storage conditions eventually killed all the pollen grains, the death rate apparently differed for the two types, the waxy pollen surviving conditions lethal to the non-waxy. Kempton suggested that waxy pollen was less mature at anthesis and that storage caused it to mature, at the same time causing non-waxy pollen to lose its vitality.

In order to explain the variation of waxy kernels from the theoretical, Mangelsdorf and Jones[86] postulated the presence of a gene *Ga* which in dominant form stimulates pollen tube growth and occurs on the same chromosome as gene *Wx*. Thus the combination *Gawx* would produce an excess of waxy seeds, while the combination *gawx* would produce a deficiency. This view has been questioned by Brink[21] and by Chao.[30] The latter found a significant deficiency of glutinous kernels from the 3 : 1 ratio in rice crosses amounting to 8·52 times the probable error and showed that in this case the theory of an accessory factor like *Ga* would not apply. He attributed the deficiency to an accumulation of many small deviations all in the same direction. Factors eliminated as causes were unequal numbers of pollen grains, faulty classification of kernels, selective fertilisation, seasonal infertility and zygotic lethals.

Sprague[116] observed germination and pollen tube growth of different kinds of pollen on corn silks under the microscope. Growth rates of the pollen tubes were practically identical, but there was a significant difference in the time required for germination and establishment of pollen tubes in the silk. Thus waxy pollen (*wxSu*) required 3·8 minutes longer, sweet pollen (*suWx*) 0·3 minutes longer, and waxy-sweet pollen (*wxsu*) 31·5 minutes longer for establishment of the pollen tube than did ordinary

pollen (*WxSu*). This work has limited the crux of the problem to a definite stage in pollination, but the cause of the differences in behaviour at this stage remains uncertain.

Eyster[51] has reported cases of extreme variation in certain strains of waxy maize. Plants heterozygous for waxy endosperm gave rise to F_2 progenies with 5-48 per cent. waxy kernels (theor. 25 per cent.) and to backcross progenies with 39-90 per cent. waxy kernels (theor. 50 per cent.).

Studies on the waxy gene, *wx*, in millet[80] and its allelomorphic gene, wx_a, in barley[63] and Argentine waxy corn[4] show them to follow a simple Mendelian inheritance and to be completely recessive to the non-waxy condition. However, crosses between Argentine waxy (wx_a) and Chinese waxy (*wx*) maize indicated partial or incomplete dominance of wx_a over *wx*. Differences in iodine colour could be observed in reciprocal crosses but these were not sufficiently clear-cut to allow accurate separation of phenotypes in the F_2 generation.

The effect of the waxy gene on kernel characteristics has in many cases been studied quantitatively, and numerous differences between waxy and non-waxy cereals reported. Some of these are fortuitous and disappear when the two types are grown under identical conditions from similar genetic stock. Other differences remain under these conditions and are considered as attributes of the waxy character.

Moisture content.—The first crop of waxy corn harvested for semi-commercial milling arrived at the processing plant with a moisture content of 20·8 per cent. as compared to 16·4 per cent. for non-waxy corn grown the same year. The waxy starch isolated from it seemed to come to equilibrium at a higher moisture content than non-waxy.[113] Further data are needed before the higher moisture retention of this one crop can be ascribed to waxy corn in general. According to Andrew *et al.*[5] waxy kernels throughout the growing period had a slightly higher moisture content than non-waxy although the difference was not statistically significant. Sweet corn kernels, 70 days after pollination, had 10 per cent. higher moisture content than ordinary corn.

Kernel weight.—As with several other characters, there is a gradation in kernel weight from ordinary corn to waxy corn to sweet corn. This is shown by the results of several investigations summarised in Table XIII. No difference in specific gravity of the three types was found.[27] The waxy gene apparently allows a slightly less complete development of the endosperm than does the non-waxy gene.[38, 73, 85]

Fat content and quality.—The fat content of waxy corn is con-

sistently higher than that of non-waxy from similar genetic stock. Abegg[1] showed that this difference is localised in the endosperm tissue where both the content of oil and its quality (based on titratable acidity) vary (Table XIV). A similar condition is found in sweet corn ; the amount of oil in the embryo is equal to that of ordinary corn but the endosperm contains significantly more. According to Abegg[2] the waxy gene increases the fat content of corn endosperm by about 0·4 per cent., while the sugary gene (*su*) increases it about 1·6 per cent. When both *su* and *wx* are present, their effect is additive (Table XV). Differences in

TABLE XIII

Effect of Waxy Gene on Kernel Weight of Corn

Type of Corn	Relative Kernel Weights, %			
	Collins & Kempton[38]	*Mangelsdorf*[85]	*Kiesselbach*[73]	*Andrew et al.*[5*]
Ordinary .	100	100	100	100
Waxy . .	95·2	96·5	96·8	98·5
Sweet . .	77	88·5	—	76·1

TABLE XIV

Effect on Waxy Gene on Oil Content and Quality

	Whole Kernel	*Endosperm*	*Embryo*
%Crude Oil :			
Waxy corn . . .	7·30	1·50	34·3
Non-waxy corn . .	6·50	1·05	34·0
Acid number of oil :			
Waxy corn . . .	34·0	82·0	4·1
Non-waxy corn . .	13·0	45·0	4·2

TABLE XV

Effect on Waxy Gene on Quantity of Endosperm Oil

Type of Corn	*% Oil in Endosperm*
Ordinary . .	1·01
Waxy . . .	1·43
Sweet . . .	2·61
Waxy Sweet .	3·00

the fat content of waxy and non-waxy pollen did not appear to be significant (0·67 and 0·64 per cent., respectively). This data has led to the speculation that the waxy gene modifies the activity of the enzyme lipase. The higher oil content of waxy corn, which was

* Calculated from their Table VI.

at first[83] considered advantageous due to its value as a by-product, has actually been of little consequence to millers because only the germ is processed for oil. The yield during milling on a commercial scale has been no higher than for ordinary corn.[110, 113] When the whole corn is used for feeding purposes, however, the higher oil content might be desirable. The oil content of Argentine waxy corn (5·4 per cent) was intermediate between that of Chinese waxy (6·4 per cent.) and ordinary corn (4·8 per cent.).[4]

For purposes of comparative analysis, the waxy gene has been introduced into several inbred lines of corn which were grown

TABLE XVI

Effect of Waxy Gene on Oil, Protein and Solubles in Corn

Inbred Line	*Difference in % of Constituent (Waxy minus Non-waxy)*		
	Oil	*Protein*	*Water-Solubles*
Dent Corn 1	0·2	0·6	0·5
Dent Corn 2	0·2	−0·4	1·9
Dent Corn 3	0·5	−1·5	0·3
Dent Corn 4	0·2	0·5	2·2
Dent Corn 5	0·8	0·7	1·8
Popcorn 1	0·3	0·2	2·0
Popcorn 2	0·1	0·2	1·8
Flour Corn	0·3	−0·2	1·3
Flint Corn	0·2	0·8	0·9
Average Difference	0·31	0·1	1·4
Average % of Constituent	3·7	12·9	7·4

under comparable conditions and differences between waxy and non-waxy lines investigated (Table XVI). The oil content of the waxy kernels was significantly higher by 0·3 per cent. (Sprague and Brimhall, unpublished).

Protein content and quality.—The data in Table XVI would indicate that the waxy gene has no significant effect on protein content. There is evidence, however, that it does affect the quality. Tadokoro[125] studied the protein fractions of glutinous and common rice and found no significant differences in the two types with respect to the fractions soluble in water, in 10 per cent. salt solution, and in 70 per cent. alcohol. After extraction with alcohol and salt solution, the protein soluble in 0·2 per cent. sodium hydroxide was isolated (oryzenin). The oryzenin from glutinous rice contained more carboxyl groups and fewer amino groups, had less argenine- and lysine-nitrogen but more histidine- and cystine-nitrogen, combined with more iodine, had a

lower optical rotation and a lower acid-binding capacity, and was more rapidly decomposed by pancreatin than oryzenin from common rice. Both had the same amounts of tryptophane and tyrosine. This work may have a bearing on the observation[27, 113] that in the wet-milling of waxy corn the starch separated less readily from the gluten.

No difference was found between the alcohol-soluble protein from glutinous and common kaoliang (sorghum).[101] During germination of rice seeds, glutelin and hordein decreased, soluble nitrogen increased, and albumin and globulin fluctuated from one species to another, but no specific differences between glutinous and non-glutinous varieties were mentioned.[64]

Feeding quality.—Waxy corn is suitable for incorporation into hog or chicken feed. Hanson[55] fed two lots of ten pigs each, free-choice, with waxy corn in one case and ordinary commercial corn in the other, both lots being provided with a supplement. After four months the response of the pigs fed waxy corn was fully equal to that of the control group. A third lot of pigs provided with both types of corn showed a marked preference for the non-waxy type. Mussehl[99] found that waxy corn was at least equivalent to No. 2 yellow dent in rations for growing chicks, and might even have a slight advantage.

However, feeding tests with rats[114] indicated that proteins of commercial corn are superior to those of waxy corn (Table XVII). Rats were fed rations containing the designated protein source at the 5 per cent. level, and germ was ether-extracted to remove oil previous to feeding.

Enzymes.—That the waxy gene exerts its influence on starch synthesis through the media of specific enzymes is a logical hypothesis. A. Meyer[87] believed that the enzyme responsible for synthesis of red-staining starch was weaker than that forming ordinary starch and obtained some evidence for this view by preparing enzyme extracts from glutinous and non-glutinous varieties of sorghum and rice. When applied to potato starch pastes, the extracts from the glutinous cereals were less potent in bringing about hydrolysis. Brink[20, 21, 22] proposed that the waxy gene influenced the type and amount of amylase formed. However, apart from the higher activity of amylase from non-waxy pollen mentioned previously, there is little to support this view. Amylase extracts from both kernels and seedlings of waxy corn hydrolysed starch pastes at the same rate as similar extracts from non-waxy corn, and the endosperm of both types was utilised at the same rate during germination, judging from gain in weight of the seedlings. Bernstein[10] claimed that α-amylase activity is entirely

absent in the developing maize endosperm. Determination of β-amylase activity in the endosperm of waxy, flint, floury, sugary and dent corn at different stages of maturity gave values for waxy which were consistent with the other varieties examined and revealed no differences bearing on the deposition of red-staining starch. According to Hanes[54] amylase is concerned chiefly with digestion rather than synthesis of starch in the plant, while phosphorylase is primarily concerned in its synthesis in a reversible system. Bliss[11] followed the phosphorylase content of waxy maize kernels throughout their developmental period. The enzyme was present in largest quantity during the first stages of growth (5·1 units 16 days after pollination), then rapidly decreased to 0·28 unit at maturity. Unfortunately no comparative data on non-waxy corn were reported. K. Meyer[91] proposed that phosphorylase is a mixture of at least two enzymes, one of which causes formation of 1,4-glucosidic linkages as in amylose, and

TABLE XVII

Biological Value of Corn Proteins to Rats

Protein	*Biological Value*
Waxy corn germ	66
Commercial corn germ	84
Waxy corn gluten	44
Commercial corn gluten	68

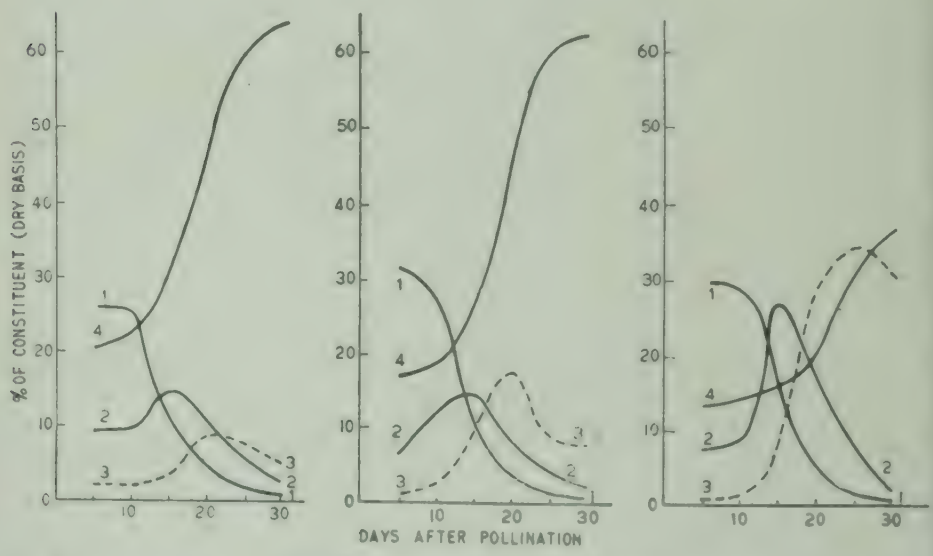
the other, of 1,6-glucosidic linkages found at the points of branching in amylopectin. If his concept is correct, both of these enzymes would be required for synthesis of amylopectin, and the enzyme system in waxy cereals could not be considered as any less complex than that of ordinary cereals.

Morphological studies.—Lampe[79] observed sections of developing corn kernels microscopically and followed the distribution of carbohydrates at various stages of growth for ordinary, waxy, sweet and waxy sweet corn. Aside from the iodine colour of the starches, no differences between waxy and non-waxy types were mentioned. In both cases starch deposition started about two weeks after pollination in the upper central portion of the endosperm and spread outward in a progressive wave. Drawings were included of endosperm cells in waxy corn at the beginning of starch formation showing small plastids at first grouped around the nucleus, then later filled with starch. These observations were confirmed by Bernstein.[10] Mudaliar[98] has compared starch formation in the developing kernels of glutinous and ordinary rice.

Carbohydrates.—In Fig. 11 : 1 are plotted the data of Culpepper and Magoon[42] comparing flint, waxy, and sweet corn at different stages of maturity with respect to their content of reducing sugars, sucrose, soluble polysaccharides (dextrin), and starch.

Curves for reducing sugars in waxy and non-waxy corn (No. 4, Figs. 11 : 1a and 11 : 1b) are essentially the same. This has been confirmed by Andrew *et al.*[5] and Bernstein.[10]

Likewise no difference in sucrose content attributable to the waxy gene is indicated (Curves No. 3, Figs. 11 : 1a and 11 : 1b). Bernstein's[10] results on endosperm are in good agreement with these figures when calculated on the same basis. The data of



(a) Flint corn. (b) Waxy maize. (c) Sweet corn.

FIG. 11 : 1.—Carbohydrates in corn kernels at different stages of growth. 1. Reducing sugars. 2. Sucrose. 3. Dextrin. 4. Starch. **(From data of Culpepper and Magoon.)**

Andrew *et al.* fail to show the maximum at 15 days, but it is possible that their material had already passed this point at the 12-day stage when samples were first collected.

The increase in starch content with progressing maturity follows essentially the same course for waxy and non-waxy corn (Curves No. 1, Figs. 11 : 1a and 11 : 1b ; see also Ref. 10). MacMasters and Hilbert,[83] in an analysis of four waxy and two non-waxy varieties of corn, showed that the former contained about 5 per cent. less starch. Likewise only two out of eight varieties of glutinous sorghum examined had as much starch as three non-glutinous varieties.

Waxy corn is characterised by a higher proportion of water-

soluble material than non-waxy. This is illustrated in Table XVI where the average difference between the two types is 1·4 per cent. These values include all material—protein, sugars, polysaccharides—soluble in water under the conditions used. It is in the amount of soluble polysaccharides, or dextrin,* that the difference occurs. Both Culpepper and Magoon (Curves No. 3, Figs. 11 : 1a and 11 : 1b) and Andrew *et al.* found a consistently higher content of water-soluble polysaccharides in waxy varieties throughout the developmental period, although the latter workers did not obtain so sharp a maximum at the 20-day stage for waxy.

Curves for sweet corn are included for comparison (Fig. 11 : 1c). In this material a large part of the carbohydrate remains in the form of soluble dextrins rather than being converted to starch. There is a higher percentage of sucrose at 2-3 weeks after pollination, although the mature sweet corn has approximately the same sugar content as ordinary corn.

Miscellaneous analyses.—Further analyses have been reported which show the range of values covered by the waxy cereals but which cannot be used for specific comparisons with the non-waxy type because of differences in environment or variety. Some of these are summarised in Table XVIII, although it should be dointed out that apparent lack of agreement may be due to pifferences in analytical procedures as well as other factors. The possibility of breeding waxy varieties low in water-solubles and high in oil and protein is indicated by these results.

The waxy corn found growing in the vicinity of Shanghai had several unique plant features which enabled it to resist drying of the silks by hot winds at the time of pollination [32]: (*a*) The upper leaves were all on one side of the plant (monostichous), (*b*) the upper leaf blades were erect instead of horizontal as in ordinary varieties, (*c*) the silks developed inside the leaf sheath before the young ears emerged. Thus the pollen was blown against the erect leaf blades and accumulated at their base. The silks were pushed into these accumulations before being exposed to air. This adaptation to dry climates was almost as interesting a feature as the waxy character of the kernels.[35] The plants were short and stocky and gave poor yields of grain with small kernels of different colours.

Because of its genetic interest, breeding work with waxy corn was continued by the U.S. Department of Agriculture and several State Agricultural Experiment Stations. Laboratory

* The term 'dextrin' as used here refers to polysaccharides soluble in cold water other than sugars. Waxy starch is not a dextrin in this sense [22, 130] and responds to the analytical methods used for determination of non-waxy starch except those estimating the blue colour produced with iodine.

TABLE XVIII

Analyses of Waxy Cereals

	Waxy Rice			*Waxy Sorghum*	*Waxy Corn*			
Investigator	*Kreusler & Dafert*[77]	*Borasio*[13, 12]	*Koolhaas*[76]	*MacMasters & Hilbert*[83]		*Schopmeyer et al.*[113]	*Andres*[4]	*Sprague & Brimhall (Unpubl.)*
Number of Samples	—	3	15	9	11	Composite Crop	—	290*
Oil, %	0·68	1·4-2·0	0·4-2·2	3·5-4·28	4·3-6·0	4·6	6·4	3·0-7·3
Protein	8·9	6·4-8·0	6·7-9·1	11·3-14·7	9·6-12·6	10·55	10·7	8·6-15·5
Ash, %	0·69	1·2-1·5	0·37-1·7	—	—	1·5	1·56	—
Starch	—	—	64·5-74·8	57-71	62·2-69	67·3	59·6	60-70
Dextrin	3·35	3-5	—	—	—	—	0·63	—
Water-Solubles	—	—	—	—	—	7·67	—	6·3-11·6
Pentosans P_2O_5*			0·18-·82				5·46	
Vitamin B^1			125-620†					

* Grown the same season in the same field from seed of all available varieties.
† In units.

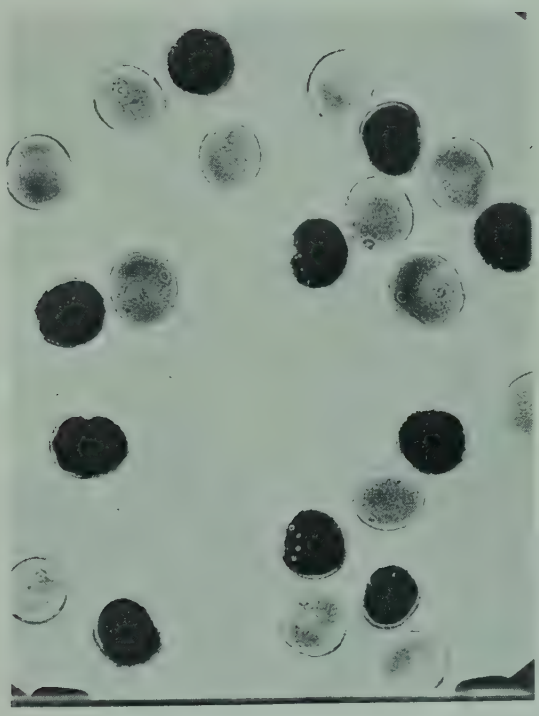


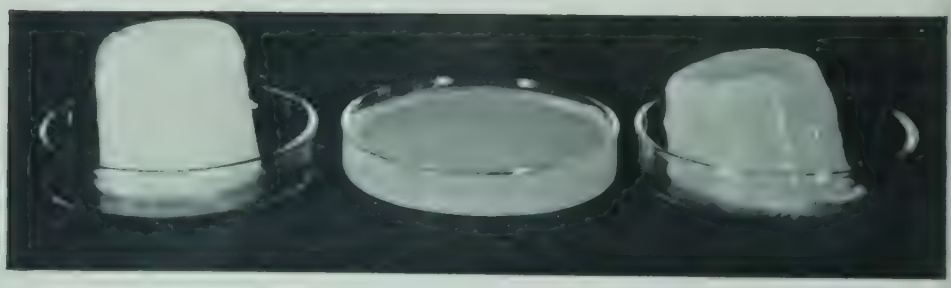
FIG. 11:2. Pollen grains from corn plant heterozygous for waxy character (Wxwx) showing differentiation by iodine stain (120 x).

(*Hixon and Brimhall*)

(*Facing p.* 270.

FIG. 11 : 3. Ears of hybrid Iowax corn.

(Hixson and Brimhall)



Corn Waxy corn Tapioca

FIG. 11:4. Gels of three starches.

(Hixson and Brimhall)

(Facing p. 27

experiments on waxy starch showed that its properties were different from any being produced commercially although it resembled tapioca starch in certain characteristics. When it became apparent late in 1941 that tapioca imports from Java would be curtailed, a concerted attempt was begun to produce waxy corn on a commercial scale. At that time only three sources of seed stock were available: (*a*) the University of Wisconsin had developed a double-cross hybrid which, however, was not adapted to the Corn Belt, (*b*) the University of Nebraska had an open-pollinated variety obtained from Chinese waxy by outcrossing with two yellow dent strains, (*c*) Iowa State College and the Bureau of Plant Industry had a small amount of seed of a double-cross hybrid known as Iowa Waxy 939, or later as Iowax 1.[60, 117] (see Fig. 11 : 3) A commercial non-waxy hybrid, Iowa 939, had been grown successfully in Iowa for several years and the four inbred lines used in its production were converted to waxy types using the backcross technique. The four waxy inbreds were then combined in a double cross to give the waxy counterpart of Iowa 939. Under normal conditions, no effort would have been made to commercialise the crop in 1942 since there was still considerable opportunity for its improvement by further breeding work. However, in 1942 enough seed was available to plant 400 acres. This was increased to 3,000 acres in 1943 and to about 20,000 acres in 1944.[110] The acreage has remained at approximately this same level for the past six years. The corn was grown by farmers under contract at a premium of about 12 per cent. The growing fields had to be segregated to prevent pollination by non-waxy pollen, which would result in loss of the waxy character, and the crop had to be shipped, stored, and processed separately to prevent contamination with non-waxy corn. It is sometimes possible to arrange the planting time so that the waxy will be fully pollinated outside the season for the common corn, since the pollinating season is of comparatively short duration. At most, the pollen from non-waxy corn penetrates only a small distance inside the field and contamination is bad only along the border rows.

The yield of commercially grown Iowax 1 in Iowa for two years was approximately equal to the State average for non-waxy hybrids. Yield tests in Nebraska[73] showed that Iowax 1 had superior vegetative characteristics and was the most productive of the waxy varieties; however, it was less well-adapted to growing conditions in Nebraska and yielded considerably lower than non-waxy hybrids developed for that State. Both Nebraska and Iowa Stations are proceeding with breeding programmes to improve

existing waxy varieties and to develop white waxy lines of corn.[72]

The glutinous sorghums have been used to some extent as a source of waxy starch. About 50,000 acres are grown yearly in the south-western States for grain and fodder. Their status has been reviewed by MacMasters and Hilbert[83] essentially as follows: most of the older varieties suffer disadvantages as a source of waxy starch, for example, Gooseneck and Chinese Amber, which are only in minor production, are usually admixed with non-glutinous varieties. Sagrain and Leoti contain a water-soluble pigment in the seed coat which is strongly adsorbed by the starch during milling to give a tan or blue-grey colour. Pearling the grain to remove the seed coat before processing partly overcomes this difficulty. However, new varieties have been produced by hybridisation in which the pigmented layer is resorbed as the seed matures and which yield white starches on milling. These are being grown in increased acreage; e.g. waxy Club (Cody) in Kansas, and waxy kafir in Texas.[125] The sorghums are largely self-pollinated and do not have to be grown in segregated plots to prevent contamination as does waxy maize. Further it is not necessary to pay a premium to the farmer for growing waxy sorghum. However, by far the largest part of the waxy starch produced to date has come from corn, because the processing companies are accustomed to handling corn and their equipment and procedures are specialised for this purpose. The new plant has been erected at Corpus Christi, Texas, especially for milling sorghum. Corn has the added advantage of keeping better in storage.

The General Foods Corporation milled waxy sorghum on a commercial scale in 1941,[83] while the American Maize-Products Company and National Starch Products, Inc., jointly carried out the processing of waxy corn in the conventional wet-milling fashion on crops from the 1942, 1943 and 1944 seasons. During plant operations, it was found necessary to feed the starch and gluten mixture on to the tables a little more slowly than with regular corn. Some difficulty was likewise encountered in obtaining satisfactorily dried starch in the continuous driers and the rate was decreased in order to bring it to the desired moisture content.[27, 113] In other respects the waxy was handled like ordinary corn and the control analytical data for processing the two were surprisingly similar.

II. The Waxy or Glutinous Starches.—The properties and uses of waxy starch have been reviewed in several recent publications.[27, 59, 83, 109, 110, 113]

Work of Dafert and of A. Meyer.—Kreusler and Dafert in 1884[77] reported the presence of red-staining starch in glutinous rice, unaware that Gris[53] had discovered it previously (1860). Dafert[44] then isolated this starch and made a study of its properties. Starches from glutinous and from ordinary rice were alike in size and shape of granules, specific gravity, percentage of carbon and hydrogen, ash content, and the fact that their pastes were precipitated by alcohol and converted to sugars by acid, saliva or diastase. They differed as shown in Table XIX. He carried out a baking experiment with rice starch, heating 2 gm. of each type in 2 ml. of water and baking at 210° C. for half an hour. The 'bread' from ordinary rice was hard and compact with a volume of only 5 ml.; that from glutinous rice had risen

TABLE XIX

Differences between Ordinary and Glutinous Rice Starch according to Dafert[44]

Property	*Glutinous starch*	*Non-glutinous starch*
Colour with iodine	Red brown	Blue
Behaviour toward chlor-zinc iodide	Swells and gives dark brown solution	Turns blue and does not swell
Temperature of complete paste formation	67° C.	73° C.
Behaviour of dilute paste with 5% tannin solution	Not precipitated	Precipitated
Optical rotation in water	+ 180°	+ 198°
Relative viscosity of 1% paste at 16° C.	2·77	1·86

beautifully to a volume of 20 ml. and was extremely porous. He prepared a dextrin solution by hydrolysing ordinary rice starch with dilute acid until it gave a red colour with iodine (erythrodextrin) and, after comparing it with dilute pastes of glutinous rice starch (erythrogranulose), concluded erroneously that the two were identical.

At the same time, A. Meyer[87] published a review on the red-staining starches including his work on glutinous sorghum starch. As compared to the ordinary variety, it was found that glutinous sorghum starch had a slightly higher gelatinisation temperature (70° C. as compared to 68°); a less marked formation of radial rifts during swelling; a more translucent appearance at equal stages of swelling; a slightly stronger polarisation cross; a greater tendency to fracture; and a greater susceptibility of the unswollen granules toward enzymes. Application of pressure to the granules immersed in water under a cover slip caused them to break into many pieces, usually pyramid-shaped with radial markings. At 30° the granules were about half dissolved by

strong malt extract in 24 hours. Fine pores were first visible at the surface, then canals, and finally they broke into layers. Numerous drawings were included showing the red-staining starch grains at various stages of swelling, iodine staining, fracture, and digestion with enzymes.

By repeated freezing, thawing and filtering of glutinous rice starch pastes, Meyer obtained a small quantity of material having a blue-violet iodine colour. Repeated cold water extraction of the unswollen granules yielded about 5 per cent. of a dextrin with no iodine colour. The main constituent he believed to be comparable to the 'amylodextrin' formed from ordinary starch by cold acid treatment. When ordinary starch was treated with 1 : 5 HCl for 2 months at 20° C., the organisation of the granules remained essentially intact since they retained a sharp polarisation cross. However, they had become very brittle, were easily fractured, and dissolved readily in hot water. This product, known as Nägeli's amylodextrin, gave a red colour with iodine in very dilute solution and a violet colour in more concentrated solution. Waxy starch upon treatment with cold acid gave a very similar product except that it imparted only a weak red colour with iodine. The theory that the iodine colour of a starch granule varies with the proportion of starch (blue), amylodextrin (violet to brown) and dextrin (brown to colourless) which it contains has already been discussed. (Much of this same material is reviewed in Meyer's book on the starch grain.)[89] Later[90] he pointed out a resemblance between waxy rice starch and glycogen with respect to iodine coloration, solubility, and precipitation from solution by tannin.

Two articles on the subject of red-staining starch in 1887[45, 88] consist chiefly in an exchange of personalities between Dafert and Meyer, although the latter presents more clearly the conflicting views on starch structure and terminology.

Floridean starch.—Publications dealing with isolated waxy starch during the period 1890-1920 are few in number. The most interesting development was the characterisation of the so-called Floridean starch, as described in several review articles.[90, 78, 102, 105] The Florideae (Rhodophyceae) are red algae which contain granules of reserve material. The granules are small and have a variety of forms, the most characteristic being shell- or cone-shaped. In some species they stain red or brown with iodine, and in others violet or blue. The staining reaction is quite varied, even for granules within the same plant. The granules seem to form at the surface of the chloroplasts rather than inside them as do starch granules in angiosperms. The

starch-like nature of the Floridean granules has been demonstrated in several ways: they are hydrolysed by acid to give a solution with a positive rotatory power from which glucosazone can be isolated; they are swelled by hot water, alkali, and chloral hydrate; they show a polarisation cross and are most easily cleaved in a radial direction; the swollen granules are solubilised by saliva or malt extract.

Glutinous rice starch.—Tanaka[129, 130] concluded that glutinous rice starch did not contain soluble dextrins since it was as completely precipitated from its pastes by barium hydroxide as were potato or common rice starches. Glutinous starch was hydrolysed to glucose by mineral acids and the products of hydrolysis with diastase were found to be the same as for ordinary starch except that possibly a smaller proportion of maltose to dextrin was produced.

Tadokoro and co-workers[124, 126, 127, 128*] pointed out certain differences in colloid-chemical properties between common and glutinous rice starch. The latter was less resistant to sulphuric acid, lower in ash content, less readily coagulated by metallic ions, higher in viscosity, and swelled to a greater extent by I-KI solution. Both starches could be acetylated to the same extent corresponding to the triacetate. Viscosity differences independent of location were demonstrated in starches from different varieties of glutinous rice. Ultra-violet light decreased the viscosity of glutinous more rapidly than common starch. According to Miyake and Ono[96] starch grains of glutinous rice are generally smaller than those from non-glutinous.

K. H. Meyer[93] reported that starch granules from glutinous rice show the same birefringence and X-ray diagram as non-glutinous. They swell at 55° C. and disaggregate at 65° C. to give a turbid suspension. The glutinous starch yields 40 per cent. of limit dextrin upon treatment with β-amylase. Its trimethyl derivative in chloroform has a lower limiting viscosity than trimethyl amylopectin from corn starch. End-group assays revealed that it is intermediate in degree of branching (6 per cent. terminal groups) between waxy maize starch and glycogen with 4 per cent. and 9 per cent. terminal groups, respectively. Ordinary rice starch upon extraction with warm water (65° C.) yields a soluble material which stains blue with iodine and crystallises out on standing (amylose). Under similar conditions, the hot-water extract of glutinous starch contains no amylose; this has been confirmed by the potentiometric iodine titration method for estimation of amylose.[7] Therefore, he concluded that glutinous

* As reported in *Chemical Abstracts*.

rice starch is composed entirely of large, branched molecules like amylopectin.*

Glutinous sorghum and barley starches.—In most respects, glutinous sorghum starch shows a remarkable similarity to waxy corn starch; e.g. in microscopic appearance; birefringence; iodine colour; content of nitrogen, phosphorus and ash; viscosity; gelatinisation temperature; appearance of swollen granules; and character of pastes.[83] Photographs of these granules after swelling in water at 70° C. and after fracturing into wedge-shaped fragments by pressure have been obtained by MacMasters and Hilbert.[83] Little work has been done on starch from glutinous barley. Its granules have a much wider variation of colour with iodine than do most glutinous starches[59, 83] except that from Argentine waxy corn.[119] The first potentiometric iodine titrations on small samples (40 mg.) of glutinous barley starch indicated a complete lack of amylose.[7] However, after the effect of fatty acids on absorption of iodine by starch was recognised,[108] the titration was repeated using defatted starch (200 mg.). Under these conditions, glutinous barley starch was found to contain 3·5 per cent. amylose, while pure waxy corn-starch contained none.[119] The original fat content (by hydrolysis) of the barley starch was 0·35 per cent. as compared to 0·08 per cent. for waxy corn starch.

Waxy maize starch.—Investigations on the properties of starch from waxy maize were not begun until 1922 when Weatherwax[133] pointed out that it stained red with iodine and in this respect resembled the glutinous starches from other sources. Unfortunately, he described the carbohydrate as having 'a violet-red colour given only by erythrodextrin', apparently failing to realise that carbohydrates having a red iodine colour may differ widely in molecular constitution and cannot all be included under one classification. His concept is not surprising in view of the confused state of starch chemistry at that time. He further expressed the belief that geneticists should pay more attention to the chemical composition of the plants with which they work. Both East[48] and Kempton[67] objected to application of the term 'erythrodextrin' on such flimsy evidence as iodine colour and agreed that further work was necessary to establish the nature of the red-staining carbohydrate.

In 1925 Brink and co-workers began a series of investigations

* Red-staining starch, or what was believed to be its major constituent, has been variously described under the terms glutinous starch, waxy starch, erythrogranulose, erythrodextrin, erythroamylose, amylodextrin, amyloerythrin, amylopectin, Amioca, and β-amylose.

on the relationship of the waxy gene in corn to the properties of its starch.[19–23] They developed a laboratory method for starch isolation which involved treatment with 0·45 per cent. NaOH to bring about gluten separation. Later, Hixon *et al.*[58, 59] built equipment for wet-milling on a pilot plant scale and subsequently made available about 50 lb. of waxy maize starch for testing purposes. It was not until after the first semi-commercial milling run at the American Maize-Products Co. plant in 1942 that sufficient starch was available for adequate industrial testing.

That waxy starch consists of large branched molecules of amylopectin is supported by a variety of data. It shows no appreciable fractionation by the use of methods which separate ordinary corn starch into two fractions differing in molecular constitution (amylose and amylopectin). Schoch's butanol-precipitation procedure[107] yields no amylose precipitate ; K. Meyer's hot-water extraction procedure yields no blue-staining, crystallisable material.[94] Earlier methods (e.g. freezing, electrodialysis, partial pasting, etc.), although they brought about poor separation of branched and linear components, served to indicate a preponderance of the more soluble fraction in the waxy over the non-waxy starch.[22, 28, 56, 94] Dilute pastes of waxy starch can be retrograded, but only with difficulty, no insoluble precipitate being formed after 450 hours at 7° C.[28] The dried precipitate obtained by addition of alcohol to waxy starch pastes readily redissolves in water.[56] Non-waxy starch is rendered largely insoluble by either treatment due to its amylose component. Three fractions prepared from the acetate of waxy maize starch by precipitation from solution had nearly the same viscosity, indicating a high degree of uniformity for the original product.[56]

Both waxy maize starch and amylopectin stain red-brown to red-violet with iodine. They give remarkably similar curves for potentiometric titration with iodine[7] and for transmission at different wave-lengths in iodine solution.[6] Waxy maize starch was found by end-group assay to contain one non-reducing end group for each 22-29 glucose units,[56, 94] which is not significantly different from the value for amylopectin or whole corn starch[92] (see Chapter 3).

The above data prove the absence of amylose in waxy starch but could conceivably apply to short linear molecules as well as large, branched ones. However, the viscosity and osmotic pressure (acetate) of dilute solutions of this starch are in close agreement with corresponding values for corn amylopectin[52] and show these molecules to be at least three or four times the size

of amylose molecules. Further, assuming one reducing aldehyde group per molecule, the low copper-reducing value of waxy starch shows it to be composed of large molecules equal in size to those of ordinary starch.[28] Schoch's alkali lability determination gives a value of 4·1 for waxy starch as compared to 5 or 6 for corn and potato amylopectins.[107] Since the value found by end-group assay (22-29 glucose units per non-reducing terminal group) does not in this case correspond to the length of a short linear molecule, it can be interpreted as corresponding to the frequency of branching for a large molecule. The behaviour of waxy starch toward enzymes bears out this viewpoint.

Waxy and non-waxy starch are hydrolysed to the same extent by soybean β-amylase (56 per cent. ;[100] 62-65 per cent.[104]), barley diastase (60 per cent.[56]), and barley amylase (47-49 per cent.[19]), yielding maltose and a limit dextrin. The latter is believed to be similar in structure to the original amylopectin except that the branches have been digested away up to the 1,6-glucosidic linkages at the points of branching. The limit dextrins prepared from waxy and non-waxy starches using soybean β-amylase were almost identical with respect to optical rotation (154·6-156·2°.) and reducing value (Rcu = 132-133). The dextrin from waxy starch was obtained in slightly higher yield (38 per cent. as compared to 31 per cent.), was more soluble in cold water, and had a redder iodine colour.[28] Brink[21] prepared similar products under the name of α-amylose using barley amylase and found that waxy starch gave an elastic, sticky, water-soluble dextrin, while that from non-waxy starch was firm, crumbly, and difficultly soluble. The difference is probably due to small amounts of retrograded amylose in the latter which are resistant to enzyme digestion. For accurate comparison, amylopectin should be used as a source of the limit dextrin rather than unfractionated non-waxy cornstarch. Brink's results[19] indicate a somewhat more rapid rate of conversion of non-waxy starch by malt diastase as compared to waxy. He isolated maltose as the osazone in both cases but found that the intermediate products of hydrolysis of waxy starch had a consistently lower specific rotation. Experiments on liquefaction of glutinous and non-glutinous rice starch by takadiastase, saliva and diastase have been reported[127, 128, 131] but the abstracts do not indicate any clear-cut differences due to the glutinous character.

While the previous experiments were carried out in dilute solution using formation of maltose as a criterion of enzyme conversion, Pigman[104] used starch concentrations of 10-13 per cent. and followed conversion by fermentability with baker's

yeast of the sugars formed. These conditions are more comparable to commercial processes for alcohol production. Waxy maize starch was completely converted to fermentable sugars by malt amylases in the presence of yeasts. The extent of action of various amylases on waxy and non-waxy corn starches appeared to be the same within experimental error (Table XX). Under conditions used for commercial modification of starch by enzyme preparations, Schopmeyer[110] found that liquefying enzymes attacked waxy corn starch and tapioca at about the same rate, judging from the decrease in viscosity of their pastes. Common corn starch proved more resistant to the enzyme (Wallerstein),

TABLE XX

Action of Amylase on Waxy Starch according to Pigman[104]

Amylase Source	*% Conversion to Fermentable Substances**	
	Ordinary corn starch	*Waxy maize starch*
Barley malt	103	100
Malt α-amylase	100	99
Kaffir malt	99	99
Aspergillus oryzae-A	98	98
Whole pancreas	77	77
Bacillus mesentericus	55	57
Wheat β-amylase	69	70
Soybean β-amylase	64	68

probably because its granular structure was less thoroughly disrupted by the temperature used in preparing the pastes.

An amylopectin similar to waxy starch has recently been synthesised from glucose-1-phosphate by means of potato phosphorylase.[57 57a]

Starch granules from waxy and non-waxy corn are alike in size and shape[83] (photographs given in Refs. 22, 70, 113); appearance of polarisation cross, gelatinisation temperature (complete loss of birefringence 71-78° C.[83]), and X-ray pattern.[9] Upon prolonged treatment of the unswollen granules with cold acid, similar yields of Nägeli's amylodextrin are obtained. These facts indicate that crystalline areas are present to approximately the same extent in both types of starch. They prove that the presence of amylose is not necessary for the expression of these properties in the starch granule. It is likely that the amylose is rather uniformly distributed throughout the submicroscopic structure in non-waxy starch. MacMasters and Hilbert[83] have pointed out that,

* Amylases present during fermentation.

since waxy granules are more easily fractured than non-waxy, they may be damaged to greater extent by grinding, such as in the preparation of powdered starch. During steeping of the corn, prior to milling, waxy starch is more susceptible to the action of sulphur dioxide, which results in decreased viscosity of the final product.[59, 88]

Waxy and non-waxy corn starches are similar in their content of non-carbohydrate constituents, except for fatty acids and possibly phosphorus (Table XXI). The larger fat content of non-waxy starch (not removed by ordinary fat solvents) is attributed

TABLE XXI

Non-carbohydrate Constituents in Waxy Maize Starch

Constituent	% of Constituent in		Ref.
	Waxy corn starch	*Non-waxy corn starch*	
Protein	0·30	0·30	(113)
	0·20	0·27–0·56	(47)
	0·19–0·37	0·19–0·31	(83)
Ash	0·12	0·11	(113)
	0·07	0·08–0·10	(47)
	0·03–0·05	0·06–0·08	(83)
Phosphorus	0·006–0·011	0·013–0·015	(83)
	0·011	0·016–0·019	(47)
	0·006–0·013	0·017	(113)
	0·0016	0·019	(19)
	0·0025	—	(56)
Fatty Acid	0·08	0·67–0·68	(47)
	0·11	0·80	(107)
Water-solubles	0·05–0·23	0·08–0·20	(113)
pH	5·7	5·5	(113)
Gelp in $CaCl_2$	203·8°	202·6–203·5°	(47)
Elementary Analysis			(56)
Carbon	44·2		
Hydrogen	6·4		

to the presence of amylose, a small portion of which is presumably in helical form and capable of containing fatty acid molecules therein[11] (see Chapter 6, Vol. I).

It is in the properties of the paste that the absence of amylose in waxy starch becomes most apparent. The granules swell more rapidly and completely in hot water,[97] alkali, and chloral hydrate.[28] Photographs of the two types of starch at comparative stages of swelling in water show that at 70° C., the waxy granules take on a segmented, often granulated appearance, and after an hour at 100° C. they have lost their identity, while non-waxy granules appear swollen and wrinkled but intact.[89]

The hot paste viscosity of waxy corn starch is much higher than for ordinary corn starch, usually exceeds that of tapioca, and

in some instances approaches that of potato starch.[16, 59, 83, 113] Viscosity of these pastes relative to one another varies widely with the source of starch and conditions of testing. Increasing the paste concentration from 5 per cent. to 10 per cent. decreases the viscosity of waxy starch relative to tapioca.[113] This might be caused by breakdown of waxy granules due to increased crowding by one another, or by the beginnings of gel-formation in the tapioca paste at the higher concentration. Two per cent. pastes of waxy starch, however, are fairly stable, the viscosity remaining constant for an hour's time at 90° C. In low concentrations there appears to be a linear relationship between the viscosity of the paste and the proportion of waxy to non-waxy corn starch used. This apparently does not hold for higher concentrations. Schopmeyer[110] has shown that when one-half to one-fourth of the starch in a 10 per cent. waxy paste is replaced by common starch the resulting hot viscosity is higher than that of either starch alone. He further studied the effect of fruit acids on viscosity, since these are often present in food products utilising waxy starch. The starches were gelatinised (8 per cent. pastes), adjusted to varying *p*H values with citric acid and allowed to stand overnight. The behaviour of waxy starch was similar to that of tapioca—both showed marked decrease in viscosity at *p*H values higher than 5·5 and thinned out badly below *p*H 3·5 but were comparatively stable in the range between these two values. Ordinary corn starch was much less sensitive to *p*H changes. The effect of tartaric and malic acids was very similar to that of citric.

When cooled, pastes of ordinary corn starch soon become opaque and set to a stiff gel in concentrations of about 4 per cent. or above, while waxy pastes even in concentrations of 20 per cent. do not increase a great deal in consistency and remain viscous and gummy for days. This is illustrated in Fig. 11 : 4, comparing the ability of three starches—common corn, waxy corn and tapioca—to retain their form after 10 per cent. pastes of each were allowed to set in moulds and transferred to petri dishes shortly before photographing. Quantitative measurements of gel strength on pastes prepared at different temperatures and cooled showed that ordinary corn starch develops maximum rigidity at 90° C., while the maximum for tapioca and waxy corn occurs at 70° C. Values for the latter are very small as compared with tapioca, however[16] (Table XXII). Hot-paste viscosities are valuable in comparing waxy with non-waxy starches, since incipient gel formation occuring in amylose-containing starches on cooling may lead to entirely different values for relative rates of flow.

The variation in starch properties brought about by crossing

lines of corn having different amylose contents has been studied by Sprague *et al.*[118, 119] Starch was isolated from non-waxy Iowa 939, Iowax 1, Argentine waxy corn, and reciprocal crosses of each (Table XXIII). These values show that the non-waxy gene (*Wx*) is not completely dominant in its action, since viscosity and

TABLE XXII

Effect of Pasting Temperature on Rigidity

Temperature of pasting °C.	*Rigidity of Starch Paste*		
	Waxy Maize 8%	*Tapioca* 7%	*Corn starch* 6%
61		10	
63		350	
67	0	475	
70	85	450	
75	0	315	0
85		110	50
90		50	450
95			300
100			110
120			15

TABLE XXIII

Effect of Genotype of Corn on Starch Properties

Corn	*Genotype of Endosperm*	*Viscosity**	*% Amylose*†	*Rigidity 5% paste*
1. Iowa 939 (1943)	WxWxWx	24·4	28·7	222
1 × 2	WxWxwx	41·8	28·7	185
2 × 1	Wxwxwx	66·6	23	61
2. Iowax 1	wxwxwx	490	0	0
3. Iowa 939 (1944)	WxWxWx	33	27·7	
3 × 4	WxWxwx$_a$	52	27·4	
4 × 3	Wxwx$_a$wx$_a$	78	26·7	
4. Argentine waxy	wx$_a$wx$_a$wx$_a$	175	2·4	
5. Argentine waxy	wx$_a$wx$_a$wx$_a$	31	2·4	
5 × 6	wx$_a$wx$_a$wx	42	1·3	
6 × 5	wx$_a$wxwx	60	0·65	
6. Iowax 1	wxwxwx	65	0	

rigidity methods are sensitive enough to differentiate starches from corn bearing none, one, and two waxy genes in the presence of *Wx*. The potentiometric iodine titration method is less sensitive but will detect a depressing influence on amylose content due to the introduction of two waxy genes (wxwxWx). Crosses

* Viscosity values are comparable only within each set of four.
† Determined by potentiometric iodine titration using defatted starch.

between Argentine waxy and Iowax 1 show no particular dominance of either parent. Results on the series Iowax 1 (no amylose), Argentine waxy (2·4 per cent. amylose), and ordinary corn starch (28 per cent. amylose) indicate that the higher the amylose content the higher the gel strength and the lower the hot-paste viscosity. This generalisation has only limited application, however; for example, pea starch with 75 per cent. amylose has relatively low viscosity and gel strength because the granules are extremely resistant to swelling by hot water.

Although a small percentage of amylose is usually present in samples of commercially milled waxy starch, this is due to contamination by ordinary starch as is readily seen by microscopic examination. When iodine is added, the presence of a few deep-blue granules in the midst of those staining reddish-brown is observed. On the other hand, the amylose content of Argentine waxy starch is not due to contamination. None of the granules stain very brown or very blue, but all appear to contain small amounts of amylose.

The characteristics of waxy starch pastes which make them particularly desirable commercially are clarity, high viscosity, lack of tendency to gel or to retrograde, adhesiveness, and ease of modification by chemicals or enzymes. The starch is known industrially as Amioca.

Dextrins and adhesives.—Amioca is readily dextrinised by heat, the conversion taking place at somewhat lower temperatures than with other starches. The dextrins form adhesive films comparable to commercial tapioca dextrins in bonding strength, clarity and remoistening properties.[26] Because of their high fluidity, Amioca dextrins are particularly useful for envelope gums which must flow freely in concentrations of one part water to two parts dextrin. Such products cannot be made from corn starch and can be made from tapioca only by a skilled operator.[110] Amioca is easily modified by alkali and can be used as a plywood adhesive, but in this instance it has no particular merit over low-grade tapioca.

Application to paper and textiles.—The thin-boiling starches made by treating Amioca below the gelatinisation temperature with acid have the advantage of remaining soft and fluid when cooled, in contrast to the stiff, brittle gels of thin-boiling corn, potato or tapioca starches.[27, 110, 113] Thus Amioca has been found particularly useful in sizing high-quality cotton goods. The goods sized in this manner dried more readily than those sized with potato or tapioca and as a result could be run faster on the slasher. The filming properties are excellent and there is a

minimum of shattering of starch from the thread. For coating textiles or paper, thin-boiling Amioca products are very satisfactory due to high adhesive strength and clarity, where a colourless transparent film is desired. Because of the cheapness of such modifications, Amioca should have an excellent future in this field, especially since thin-boiling non-waxy corn starches have very poor adhesive strength and are of little value in paper coating. Patents[111, 112] have been issued for thin-bodied waxy starches prepared by treatment with dilute sulphuric acid or an oxidising agent like sodium hypochlorite for use as adhesives, sizing agents, or coating ingredients on paper. Such products may have application for the coating of tracing cloth where clarity of film is important, and for laundry starch because of their penetrating power.

TABLE XXIV

Effectiveness of Various Starch Products in Sizing Paper Sheets[71]

Product used in sizing	*Increase in bursting strength of paper sheets*
Pregelatinised waxy corn starch	18·3
Amylopectin	10·4
Pregelatinised non-waxy corn starch	7·2
Whole cooked corn starch	5·1
Amylose	0

Waxy corn starch, pregelatinised by passing through heated rolls, has been found very effective in sizing paper sheets[71] (Table XXIV).

Food products.—In food products where a prepasted starch is desired, such as the minute tapioca type, Amioca is excellent because of its high water-absorptive power, and the fact that the gelatinised dried starch will hold together well. Common corn starch cannot be used for such products since the gelatinised dried pellets become horny and will not redisperse in water. Amioca is very promising as an emulsifying or suspension agent in salad dressings, creamed soups, sauces, gravies, and canned foods, where it imparts a highly desirable, smooth, creamy texture.[71, 113] It is admirably suited for use in pie fillings because of high viscosity, clarity and lack of syneresis.[110] The thin-boiling waxy starches have sufficient fluidity and solubility for use in mixtures with sugars to give body to candy or jelly products.

Andrew *et al.*[5] investigated the canning quality of waxy corn. When canned 18 days after pollination, non-waxy corn was very mealy and unsuited for eating purposes. Waxy corn gave a thick, viscous, unpalatable product which resembled tapioca

and could not be poured from the can (see also Ref. 42). However, waxy sweet corn was judged slightly better in canning quality than ordinary sweet corn, although the two were quite similar.

When conditions are properly modified, waxy corn can be used to prepare hominy which compares favourably with ordinary hominy in appearance, size, flavour, and palatability.[82] The waxy corn requires a weaker lye solution, a shorter heating time in the lye for removing hulls, and a shorter boiling time for swelling. If these precautions are not observed, the kernels may partly disintegrate and lye removal becomes almost impossible. Preliminary experiments by Lowe[82] showed that waxy cornmeal puddings were superior to those made with regular cornmeal although their consistency was more rapidly reduced by fruits or fruit juices. On the other hand, puddings made from unmodified waxy starch were less desirable in both consistency and flavour than ordinary corn starch puddings. When 5-15 per cent. of the flour was replaced by waxy corn starch in making angel cakes, a significant decrease in both flavour and volume was observed.

Addition to drilling muds.—Kerr[71] found roller-dried waxy starch to be three or four times more effective than the commercial starch products now used in preventing water loss from oil-well drilling muds.

The application of waxy starch for a wide variety of uses is still in the early stages of development. It is to be expected that a starch which is relatively pure amylopectin will find special uses for which it is more suitable than any of the present commercial starches.

The preparation of this review was facilitated by a grant from the American Maize-Products Company. Dr. G. F. Sprague kindly permitted the use of the photographs in Figs. 11 : 2 and 11 : 3.

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Chapter 12

DEXTRINS AND DEXTRINISATION

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The term ' dextrinisation ', as used in this chapter, refers to the modification of starches induced by subjecting them to heating in a substantially dry commercial state. The starch may previously have been sprayed with relatively small amounts of aqueous acids, fumed with hydrogen chloride or chlorine, or blended with various chemicals. Or, as in the case of a true ' British gum ' conversion, it may remain untreated prior to conversion. But the broad characterisation of a dextrin is that it has been produced by *dry heating*, as distinguished from the sundry wet processing techniques to produce types of thin-boiling starches. Such is the usual meaning of dextrinisation, to a commercial starch chemist or technologist.

There are two principal physical manifestations of dextrinisation which have a critically important commercial significance. They are :

1. Aqueous Flow.
2. Aqueous Solubility.

(1) *Flow.*—Dextrinisation if manifested in two types of aqueous flow : (*a*) Non-Newtonian ; (*b*) Approximate Newtonian.

Typical non-Newtonian flow is graphically illustrated in Fig. 12 : 1 representative of the viscosity-temperature relationship.* of a ' white ' corn dextrin converted under relatively mild heating. Deviations from linearity are noticeable, even at 10 per cent. solids concentration, and very pronounced at $33\frac{1}{3}$ per cent. The upcurve at room temperature is probably caused by association through hydrogen-bonding.[1] The curves of Fig. 12 : 1 are

* The log of the viscosity in kinematic units is plotted against reciprocal of absolute temperature in accordance with the fundamental relationship

$$\eta = Be^{E_{vis}/RT}$$

in which the plot of log η vs. $1/T$ is linear for an ideal or Newtonian fluid. Deviation from Newtonian flow is very manifest in this method of plotting.

characteristic of non-Newtonian flow and are typical of dextrins with ' starchy ' properties.

Fig. 12 : 2 illustrates an approximation to Newtonian flow. The solid lines represent a moderately soluble (at 25° C.) British gum dextrin, the dotted lines, a completely soluble envelope seal-

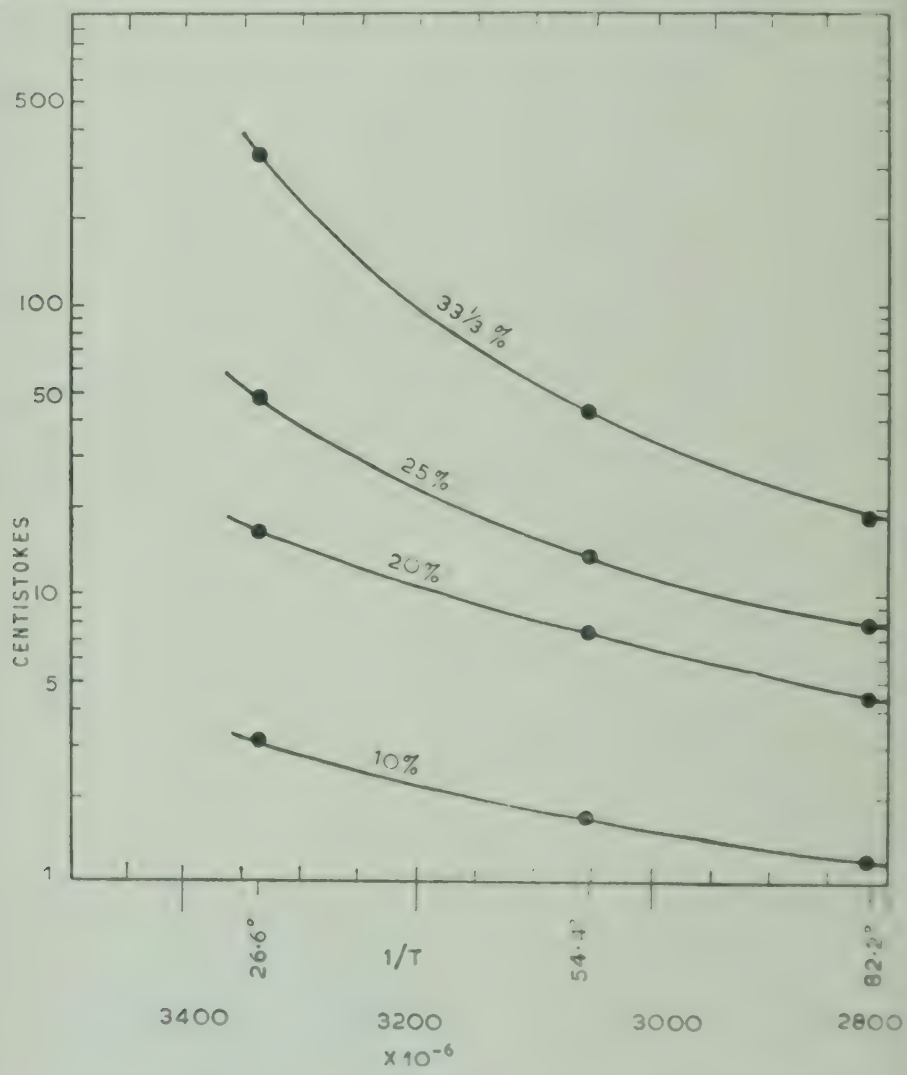

Fig. 12 : 1.

type dextrin. Both dextrins were converted at higher temperatures than the dextrin of Fig. 12 : 1.

Mild heating thus is characterised by more or less pronounced non-Newtonian flow, strong heating inducing approximation to ideal or Newtonian flow.

(2) *Solubility.* Dextrinisation promotes aqueous dispersion to a more or less transparent ' solution ' of colloidal character.

'Solution' may be brought about either at room temperatures or by cooking. The technical term 'solubility', as applied to a dextrin, commonly refers to its filtrable dispersion in water at 25° C., and is necessarily determined within a low concentration range. In general, the higher the solubility at room temperatures,

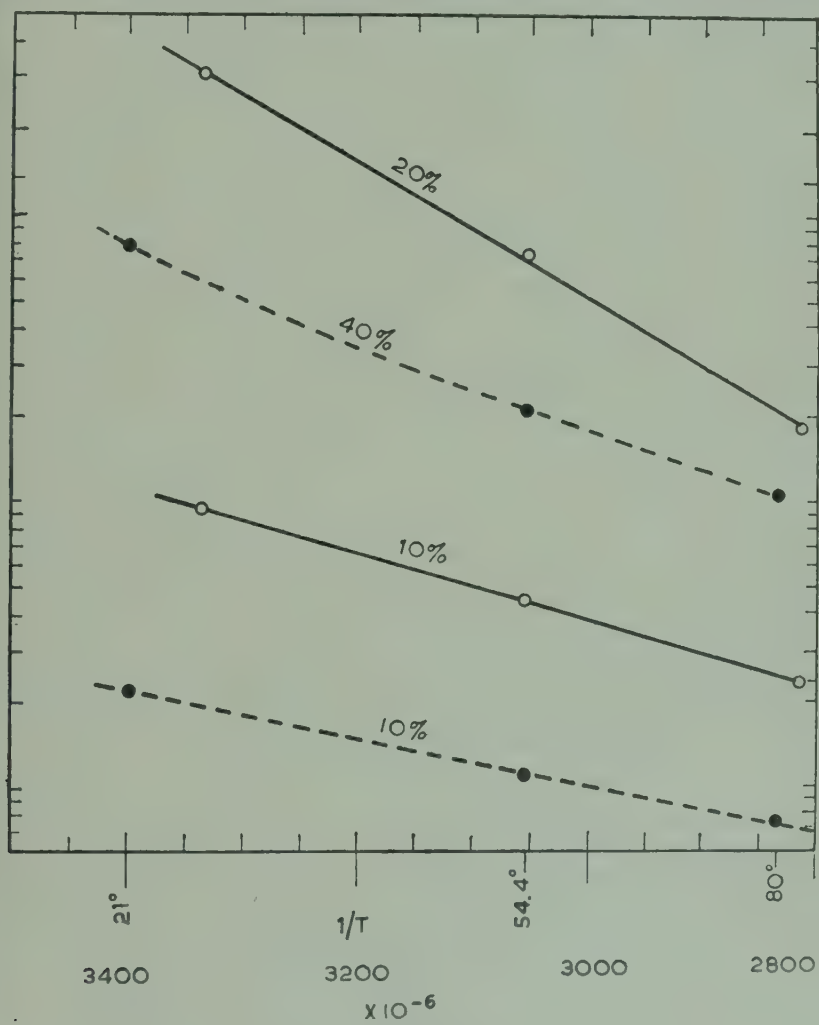

Fig. 12:2.

the nearer the approach to Newtonian flow. Low-soluble 'white' dextrins may yield quite transparent low-viscosity 'solutions' at *c.* 80° C. but they thicken rapidly and cloud or 'revert' with falling temperature, *also upon ageing at all temperatures*, a characteristic phenomenon which is especially pronounced at commercial concentrations. By contrast a completely cold-water soluble dextrin usually exhibits good viscous stability and clarity.

The clarity of a dextrin gum ' solution ' at working concentrations is an important index of its commercial utility, provided that it retains its clarity and does not increase too appreciably in viscosity over reasonable periods of time. Film-forming homogeneity is a function of clarity which in turn is a function of ' solubility '. These desiderata in general are more nearly approached by strong heating than by acid hydrolysis.

So far we have been dealing with the physical effects of dextrinisation. These are subject to graphical demonstration and are well known to experienced starch chemists. But the causal background of these effects is still, unfortunately, obscure and speculative. Chemical tools with which to probe the complex structural changes involved in dextrinisation have been scarce, and most of them are suspect from the point of view of polymer degradation. Starch and its modifications, as well as cellulose, are very readily degraded through hydrolytic scission of glucosidic linkages. When a chemist has tinkered with a starch, or some modification of it, for example, through methylation or acetylation, the question always obtrudes: is the chain polymer still present in its original degree of polymerisation ($\overline{DP}$)? Of all methods of substitution, the acetylation technique in pyridine is thought more nearly to reflect the original $\overline{DP}$ of the treated material.[2]

Nevertheless since it has been shown[2] that the granular organisation should first be destroyed—with the consequent probability of considerable damage to polymer structure—it can hardly be denied that this ingenious technique is also suspect. Moreover, the temperatures and time involved for complete acetylation are usually considerable.[2]

So far we have been dealing with the physical effects of dextrinisation as revealed in viscous properties. T. C. Taylor's method of determining what he called ' alkali lability '[3] represents the first really significant chemical probing of the complex structural changes occurring in the modification of starches by aqueous acid and heat. The sample is digested in aqueous alkali, excess iodine added, and iodine absorption per 100 mg. of sample ultimately determined. If a curve be plotted of iodine absorption versus time of conversion, the iodine absorption first rises to a peak and then falls, this phenomenon being particularly marked at the high conversion temperatures used for the production of British gums and ' Canary ' dextrins. The observed fact that the peak in alkali lability coincides with sharp inflections in both the solubility and viscosity curves[4] is suggestive of structural changes which T. C. Taylor originally postulated as the probable forma-

tion of inner ether anhydrides,[5] a result subsequently reported by Brimhall.[6] Since viscosity is related to degree of polymerisation ($\overline{DP}$), and a sharp inflection in the viscosity curve, as a function of conversion time, coincides with the alkali lability peak,[4] it seems probable that new cross-linkages are initiated at this point through ether bridges between primary chain fragments. The significance of alkali lability data is still admittedly speculative, but from considerable experience in applying the method the author is of the opinion that the alkali lability curve of a starch conversion may possibly reflect the degree of branching or cross-linking. When the curve is rising, the starch is being stripped of branched chains; when it is falling, branching, or rather cross-linking through inner ether anhydrides is proceeding. Points of branching should tend to check the reaction of the aqueous alkali along the unbranched chain polymers (a well-converted soluble canary dextrin being relatively stable to aqueous alkali). The alkali lability peaks may thus reflect an equilibrium of hydrolytic scissions and the formation of a new type of branching or cross-linkage, the relative peak heights on different dextrins bearing a direct proportion to the degree of hydrolytic scission. The hypothesis would seem worth investigating.

During the last decade, molecular weight (M) and $\overline{DP}$ determinations of high polymers have assumed increasing theoretical and practical importance. The ultra-centrifuge, osmometry, and light-scattering have been applied to the problem. The relationship between 'intrinsic viscosity' (η) and M [or $\overline{DP}$] has also been established on a modification of Staudinger's formula as:

$$[\eta] = K\,M^a \text{ (or } K\,\overline{DP}^a) \qquad (1)$$

(η) being a dimensionless quantity established by extrapolation to zero concentration of the plot of η_{sp}/c against c, where c is concentration and

$$\eta_{sp} = \frac{\eta_{\text{solution}}}{\eta_{\text{solvent}}} - 1$$

From formula (1) a plot of log $[\eta]$ *vs* log M (or $\overline{DP}$) should be linear.

Numerous papers have been published along these lines on high polymer solutions of low concentration, inclusive of esters of amylose and amylopectin starch fractions, with, unfortunately, considerable disagreement to date. The techniques of molecular weight determinations are still admittedly difficult, requiring

prolonged experience and the development of improved apparatus. Osmotic pressure measurements are perhaps most widely popular, and intrinsic viscosity $[\eta]$ is useful where K and a are known *accurately*. Unfortunately there is a wide gap between the lower limits of accuracy of osmometry (ca. M = 10,000) and the upper limits of cryoscopic measurements, and it is in this range that highly modified starches and dextrins probably lie. The author and co-workers attempted to bridge this gap with an adaptation of the Barger isopiestic method[7,18] and obtained molecular weight data on nitric esters of starches, thin-boiling starches, and dextrins, which subsequent unpublished investigations have led us to conclude are probably low, particularly for the ' pyro-dextrin ' esters.

Investigations by other workers of the molecular weights of *commercial* modified starches and dextrins would seem urgently needed, for in such studies lie the clues to the structural changes involved in dextrinisation and rationalisation of the physical properties of these products, and improvements in manufacturing methods. The new light-scattering method may prove to be applicable to this relatively low range of molecular weights. The author would definitely rule out aqueous dispersions of starches and dextrins for molecular weight determinations on account of the probability of association, even at extreme dilution. It is believed that the best method of preparation would be to form either acetate esters after the manner of Hassid,[9] an exceptionally mild acetylation treatment, or nitric esters after Caesar,[10] using N_2O_5 in $CHCl_3$ in presence of NaF. In these mild methods of esterification, the acetyl content or the nitrogen content of the resultant products should also be of considerable significance in reflecting on the structure of the short polymer chains, appreciable decreases from the theoretical values of the triacetate (44·8 per cent. acetyl) and the trinitrate (14·14 per cent. N) bearing upon the formation of inner ether anhydride cross-linkages.

At high conversion temperatures the nitrogen contents of dextrin nitric esters[7] showed definite decreases from the trinitrate value, a result which should be further explored. The very interesting fact was also observed[7] that the nitrogen assays of esters of highly degenerated, low viscosity, aqueous acid conversions showed no tendency to rise above the trinitrate value.

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Chapter 13

THE ESTERS OF STARCH

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I. Structure of Starch.—The expression, derivative of starch, as used in this and subsequent chapters by the same author, refers to compounds or mixtures of compounds which retain the intact starch molecule to an appreciable extent. The more important of these to date are the organic esters, the inorganic esters, the ethers,[28] and the oxidised derivatives.

Although the preceding paragraph implies that the starch molecule tends to remain intact during the preparation of these several derivatives, this is only relatively true. With the techniques now in use for the esterification, etherification,[63] and oxidation of starch, there is at best some degradation of the starch molecule.

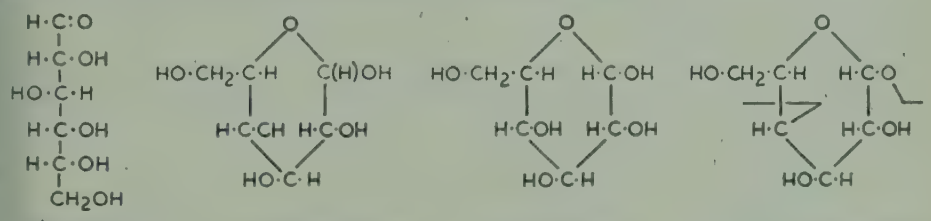
During the past, starch, because of its cheapness and commercial availability, has been used to meet demands which call for only slight changes in its chemical and/or physical properties. The per pound cost, moreover, in effecting these changes is very small. More recently consideration has been given to both more pronounced and more expensive changes in the chemical and physical properties of the starch molecule with respect to the preparation of various types of derivatives.

The discussion of the derivatives of starch, demands some consideration of the structure of the starch molecule. Although this is presented in detail in Chapter 3, Vol. I, it will be given brief consideration here.

It is now known with a reasonable degree of certainty that (1) the starch molecule is composed of D^+-glucose units (I),[63] (2) these units exist in the cyclic or pyranose ring form (II),[27] (3) the pyranose rings in starch are of the α-form (III),[27] (4) the α-forms of these glucopyranose rings are bonded together through oxygen atoms,[27] (5) the two general points of linkage are at positions 1.4 and 1.6,[110] and (6) the hydrolytic products, with increase in extent of hydrolysis, are dextrins, maltose, and D^+-glucose.[27]

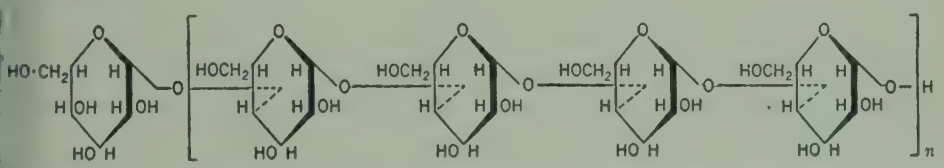
In Formulas II, III, and IV, it should be noted that a group to the right, of a carbon atom of the ring, is back of the plane of the

molecule whereas every group to the left, of a carbon atom of the ring, is in front of the plane of the molecule.



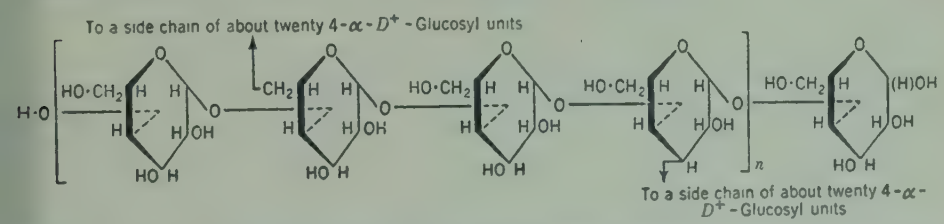
I. D^+-Glucose open chain form — II D^+-Glucopyranose α, β-mixture — III. α-D^+-Gluco-pyranose — IV α-D^+-Glucopyranosyl residue

When the units indicated by Formula IV, in which each group to the right of a carbon atom of the ring is back of the plane of the molecule whereas each group to the left of a carbon atom of the ring is in front of the plane of the molecule, are bonded together, a thread-like molecule of the type shown in Formula V is obtained :[27]



Formula V. 1.4 Linkage of α-D^+ glucosyl units in the formation of a continuous-chain starch molecule.

Recent studies definitely indicate, however, that starch is normally composed of two or more distinct varieties of molecules. The one is believed to be of the continuous chain variety, as represented by Formula V, whereas the other is of a branched chain variety, one type of which is indicated by Formula VI.



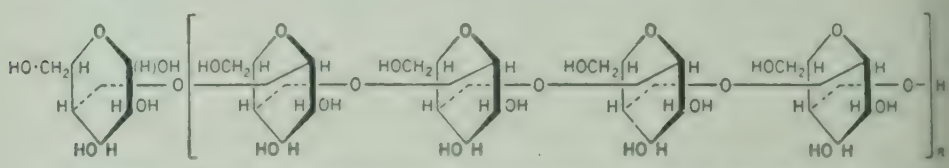
Formula VI. 1.4 Linkages of α-D^+-glucosyl units, with periodic branching, to give a branched chain starch molecule.

The available data indicate that starch is best represented as a mixture of molecules of the type shown by Formulas V and VI. The fractionation of ordinary corn starch indicates that it contains about 70 per cent. of the continuous chain variety (Formula V) and 30 per cent. of the branched chain variety (Formula VI), whereas corn starch from waxy maize is almost entirely of the type represented by Formula VI.[110]

If Formulas V and VI are approximately correct for the structure of starch, then there are two to three hydroxyl groups per glucopyranose residue which are available for esterification or etherification. Two series of derivatives, in fact, should be expected in each case and two series have been reported.

There are, likewise, hydrogen atoms bonded to the carbon atoms of the glucopyranose residue which may undergo substitution without rupture of (1) the chain structure or (2) the pyranose ring. Such derivatives have been reported, particularly in the case of the halogen derivatives.

The starch molecule differs from that of the cellulose molecule (Formula VII) in that the latter contains β- instead of α-type of linkages and is essentially of the continuous chain type.



Formula VII. Diagrammatic representation of the cellulose molecule.

It should be noted in the proposed structures for starch (Formulas V and VI) that both bondings of a given pyranose residue are back of the plane of the molecule, thus necessitating a balling-up effect, whereas in the case of the proposed cellulose structure (Formula VII) the 1-position bonding is in front of the plane of any given molecule whereas the 4-position is back of the plane of the adjoining molecule. This permits a layer-like arrangement of the glucopyranose residues in cellulose to make up a linear type of molecule.

II. Pretreatment of Starch.—The pretreatment of starch for the preparation of derivatives usually consists of (1) the removal of fatty material and phosphorus[75] and (2) the disintegration and or swelling of the starch granule.[145] The fatty material and a portion of the phosphorus may be removed by extraction with 85 per cent. methanol[111] or ethanol which contains a small amount of nitric acid.[75] The disintegration or swelling of the granule may be accomplished by use of alcohol,[59] aqueous chloral hydrate,[61] aqueous pyridine,[43] formic acid,[139] hot glycerol,[145] hydrogen chloride,[97] hydrogen peroxide,[129] phosphoric acid,[126] or water.[94]

Alcohol is frequently used to precipitate starch from its aqueous solution. In some instances the freshly precipitated starch, still wet with alcohol, has been esterified, but this is not recommended as the alcohol is esterified preferentially.[59] The starch from the alcohol precipitation may be isolated as a dry fluffy powder by

(1) pouring the starch paste into five or more times its volume of alcohol with vigorous stirring, (2) removing the remaining traces of water by filtering, stirring in fresh portions of alcohol and filtering, and (3) working at all times so as to prevent the adsorption of atmospheric moisture.[142]

The preheating of starch granules in aqueous chloral hydrate or water results in swelling, disorganisation, disintegration, and partial solution.[61] Little or no degradation accompanies this treatment.[82] Special precautions must be used during the isolation, however, or a horny, non-reactive product is obtained. Acetone may be used to precipitate the starch from the aqueous solutions of the chloral hydrate.[82]

Swelling or disorganisation of the starch granule, with little or no degradation, is effected by the use of aqueous pyridine.[43] The greatest swelling occurs in 30 per cent. aqueous pyridine.[57] A newer procedure uses 60 per cent. pyridine to swell starch, distils the water as a pyridine-water azeotrope (60 to 40 per cent. composition), and obtains an anhydrous gelatinised starch.[87] Pretreatment with formic acid transforms starch to a more easily esterifiable product but doubtless causes some chemical degradation or alteration of the starch molecule.[139] Similar effects are obtained in general by other acids, concentrated solutions of acid salts, oxidising agents, or high temperatures. Comparable results are obtained by the use of hot glycerol, hydrogen chloride,[97] hydrogen peroxide,[129] 85 per cent. phosphoric acid,[126] or water.[94]

III. Organic Esters of Starch.—The story of the historical development of the organic esters of starch is available in publications such as *Stärkeacetat* by Worden in 1913,[144] *Stärkeacetat* by Halen in 1923,[48] a paper by Gault and Ehrmann in 1924,[45] a patent review by Dejarme in 1938,[29] an historical survey by Burkhard and Degering in 1942,[18] *The Chemistry and Industry of Starch* by Kerr in 1944,[66] and *Advances in Carbohydrate Chemistry* by Pigman and Wolfrom in 1945.[96]

The types of starch which have been acetylated include banana,[51] canna.[49] corn,[62] horse chestnut,[59] rice,[6] waxy corn,[54] and wheat.[13]

Section I. Acetylation of Starch

Part A. Without the use of a Catalyst.—Acetylating reag
which have been used without a catalyst in the preparati
organic esters of *raw* starch include acetic acid,[25] acetic anhydr
acetic acid-acetic anhydride,[85] acetyl bromide,[85] acetyl chl
and ketene.[19]

When raw starch is treated with glacial acetic acid at

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C. for some hours, a product with an acetyl value of about 33·4 per cent. is obtained.[25] The treatment of potato starch with glacial acetic acid, at reflux temperature for 296 hours, gave an ester with an acetyl value of 44 per cent.[22] The product known as 'feculose' is obtained by the treatment of starch with glacial acetic acid at 90° C.,[134] and acetylation may be effected even at 80° C.[133]

Raw starch was first acetylated in 1865 by treatment with acetic anhydride.[112] Two products were obtained, one of which was soluble in both acetic acid and alcohol but insoluble in water, whereas the other was insoluble in organic solvents but soluble in water. Both were hydrolysed to yield a dextrin.[112] Four years later starch was treated with acetic anhydride at 140° C. to give an ester, which was hydrolysed to regenerate the starch.[112] In the following year (1870) Schuetzenberger reported that at 140° C. the principal product is a triacetyl starch whereas at 150° C. a triacetyl derivative of *soluble* starch is obtained.[112] The literature indicates also that the treatment of *raw* starch with acetic anhydride (*a*) at room temperature yields the acetyl derivative,[93] (*b*) at 70° C. yields maltose octaacetate as one of the products,[127] and (*c*) at 90° C. gives 'feculose' as the principal product.[134] The rate of acetylation of wheat starch by use of acetic anhydride at 35° C. has been studied.[11]

Acetic acid-acetic anhydride mixture, as an acetylating agent for *raw* starch, was used by Michael in 1883.[85] According to patent claims this mixture on starch, either with or without pretreatment with acetic acid, is much more effective as an acetylating agent than is acetic acid alone.[17] When *raw* starch is refluxed with acetic acid-acetic anhydride for 4 hours, a product with an acetyl value of about 36 per cent. is obtained.[18] Thereafter the rate of acetylation decreases appreciably and as much as 45 hours is required to obtain the triacetyl ester.[18]

One procedure treats 25 gm. of dry raw starch with 45 ml. of acetic anhydride and about 100 ml. of glacial acetic acid and then refluxes the mixture for a number of hours until the desired amount of esterification is effected.[19] The rate of acetylation is very slow until an acetyl value of about 4 per cent. is attained, then very rapid, and finally slow but uniform.[19] The marked increase in the rate of esterification is accompanied by a clearing of the viscous solution. The reaction product is precipitated by pouring into cold water, then leached for 24 hours, filtered, washed, and dried.[19]

Acetyl bromide and acetyl chloride were both used as acetylating reagents for raw starch in 1883.[85] These reagents, when

used alone[85] effect both acetylation and degradation of the starch molecule. Ketene may be used to acetylate raw starch if only a low degree of esterification is desired.[19]

Other studies on the acetylation of raw starch, without a catalyst, include the preparation and properties of the esters of starch,[134] the determination of the heat of esterification,[64] the formation of diesters with subsequent degradation,[101] and the production of esters which are soluble in both chloroform and tetrachloroethane.[121]

Both acetic acid and acetic anhydride have been used, without a catalyst, to prepare acetyl derivatives of pretreated starch. The pretreatment may be with alkali, ammonia, formaldehyde, or steam.[33] Acetic acid *vapours* may be used as the acetylating agent.[33]

Part B. With the use of a Catalyst.—Acetic acid, with catalysts such as sulphur dioxide[32] or sodium acetate,[22] has been used at the reflux temperature of the mixture for the acetylation of *raw* starch. Sodium acetate is a weakly positive catalyst for this esterification.[19]

Acetic anhydride has been used for the esterification of *raw* starch in the presence of catalysts such as halogen acid,[114] pyridine,[15] sodium acetate,[68] sulphuric acid,[115] sulphur trioxide,[71] and zinc chloride.[135] The use of halogen acid as a catalyst results in rather extensive degradation.[114] Starch is dissolved readily by acetic anhydride, saturated with dry hydrogen chloride, but it is converted in part at least into low molecular weight, chlorine-containing products, such as acetochloromaltose.[7] Acetobromoglucose may be obtained in 85 per cent. yields by the treatment of raw starch with acetic anhydride, which is saturated with hydrogen bromide.[5] The use of pyridine as a catalyst yields the triester[81] and effects less degradation than does the use of sulphuric acid.[15] Sodium acetate, when used in acetic anhydride under reflux for four days, catalyses the esterification of raw starch and gives a non-uniformly acetylated derivative (43·5 per cent. acetyl value).[68] The rate of acetylation, moreover, is very slow.[11] The use of sulphuric acid as a catalyst, other than in low concentrations and at low temperatures, results in some acetolysis to yield esters of more than theoretical acetyl value as well as some pentaacetyl glucose.[115] The rate of esterification is increased by an increase, within limits, of the amount of sulphuric acid.[19] Sulphur trioxide, with mere traces of moisture, should have a catalytic effect somewhat comparable to that of sulphuric acid. Zinc chloride is less effective as a catalyst than is sulphuric acid[19] and probably causes less degradation.[135]

A mixture of acetic acid and acetic anhydride has been used for the acetylation of raw starch with catalysts such as the Barnett mixture (Cl_2 SO_2),[2] sodium acetate,[19] sulphonated acids,[31] sulphuric acid,[36] and zinc chloride.[73] Acetic acid in acetic anhydride, in the presence of equal parts of chlorine and sulphur dioxide (Barnett mixture),[2] readily acetylates starch at 55° C. to give a product with an acetyl value of 44·8 per cent.[52] When the acetic acid-acetic anhydride mixture is used with sodium acetate as the catalyst, the rate of acetylation is very slow.[19] Sulphonated fatty acids, or sulphonated salicylic acid, catalyse this esterification.[31] This mixed reagent, with sulphuric acid as a catalyst, is an effective acetylating reagent.[36] The use of sulphuric acid, however, is limited, because it catalyses the hydrolysis of starch.[19] It has been used most generally in the esterification of horny starch, which resists the penetration of esterification reagents. Zinc chloride is somewhat less effective as both an esterification and a degradation or hydrolytic catalyst.[73]

Acetyl bromide[130] or acetyl chloride,[130] in the presence of barium carbonate, effect the acetylation of raw starch. Esterification with these reagents, however, is accompanied by some degradation of the starch molecule.[130]

Ketene, in the presence of an acid catalyst such as sulphuric acid[86] or hydrogen chloride,[19] produces a low degree of acetylation of raw starch. Somewhat comparable results are obtained with ketene in the presence of a trace of chlorine.[19]

The use of catalysts for esterification of raw starch is known to effect an appreciable amount of degradation.[40] When the Barnett mixture is used,[2] for example, the product has the reducing power, solubility, and viscosity characteristics of a partially degraded starch.[50] The use of ammonium nitrate as a catalyst, at or above 110° C., gives degraded starch acetates of low viscosities.[117] Sulphuric acid was used as an esterification catalyst for the carbohydrates as early as 1879.[36] The rate of acetylation is increased by an increase in the concentration of the sulphuric acid, up to a certain limit, and by an increase in temperature.[116] Acid catalysts other than sulphuric acid are usually less effective and their use is accompanied by considerable degradation, but both sulphuric acid and phosphoric acid cause degradation of the starch molecule.[128] Potassium and sodium thiocyanates, as catalysts, are about as effective as is sodium acetate.[136] *p*-Toluenesulphonic acid is an effective catalyst but has a very long induction period.[19] Glacial acetic acid appears to increase the rate of esterification.[19] The affec :o :time varies with the type of catalyst.[19] Other catalysts which have been used include magnesium perch-

lorate,[77] sulphuryl chloride,[77] and the Twitchell reagent.[77]

Starch has been pretreated for esterification by use of acetic acid,[19] acetic anhydride,[19] alkali,[33] glycerol,[100] heat,[98] mechanical disintegration,[42] pyridine,[42] and water.[95] Acetylating reagents which have been used on *pretreated* starch include acetic acid vapours,[33] acetic acid with a trace of sulphuric acid,[19] acetic anhydride with an alkaline thiocyanate,[135] acetic anhydride with perchloric acid or thionyl chloride,[125] acetic anhydride with pyridine,[99] acetic anhydride with sulphuric acid,[52] acetic anhydride with zinc chloride,[137] acetyl chloride with pyridine,[91] and acetyl chloride with quinoline.[65]

Starch, which is pretreated, without drying, with pyridine, is acetylated smoothly by the use of acetic anhydride.[42] It is desirable, however, to start with dry starch and thus avoid the hydrolysis of the acetic anhydride by the water present in the reaction mixture.[19] The rate of acetylation, under a variety of conditions, has been studied.[66] An anhydrous starch paste may be prepared by treatment of raw starch with pyridine and subsequent removal of the water as a pyridine-water azeotrope.[92] The paste is then acetylated with acetic anhydride or acetyl chloride in the presence of pyridine.[92] Acetylation with a minimum of degradation of the starch molecule is obtained by mechanical disintegration of the starch and subsequent treatment with acetic anhydride in pyridine.[142] A pyridine-pretreated starch is acetylated smoothly by the use of acetic anhydride and a trace of sulphuric acid.[102]

Little or no modification results from the pretreatment of starch with pyridine, but extensive modification is believed to result from the use of the Barnett catalyst.[58] Acetic anhydride, in the presence of sulphuric acid, is an effective acetylating agent even at or below room temperature to give a 46·7 to 47·4 acetyl derivative from a Zullowsky heat-modified potato starch.[98] When wheat starch is gelatinised in water, precipitated with alcohol, dried, and acetylated in the cold with 6 parts of acetic anhydride and 0·05 part of sulphuric acid for 8 hours, a derivative of 46·7 per cent. acetyl value is obtained.[95] By raising the temperature to 55° C., the acetyl value may be increased to 47·3 per cent.[95] A starch, which has been pretreated in glycerol at 100 to 170° C., yields a partially degraded derivative when acetylated by use of acetic anhydride and zinc chloride at 50 to 80° C.[137]

The acetylated starches may be used in (1) the preparation of coating and livering compositions,[80] (2) the synthesis of ethers through trans-etherification,[53] and (3) the production of chlorinated acetylated starches by treatment with phosphorus pentachloride in toluene.[93]

Section II. Physical Properties of Acetyl Derivatives of Starch

The physical properties displayed by acetyl derivatives of starch are functions of the (1) kind and type of starch, (2) pretreatment of the starch, (3) degree of acetylation, (4) extent of degradation, and (5) presence of trace constituents, particularly nitrogen and phosphorus residues. Ordinary corn starch, for example, contains about 70 per cent. of the continuous chain variety and only 30 per cent. of the branched chain variety of molecule, whereas waxy maize starch is composed essentially of the branched chain type of molecule. The acetyl derivatives of ' amylose ' resemble those of cellulose much more than do the comparable derivatives of ' amylopectin '. The pretreatment of the starch determines to a considerable degree the severity of the acetylation required to effect complete esterification, and thus becomes an index of the extent of degradation. The properties of the derivative vary markedly with both the degree of acetylation and the extent of degradation. In the case of potato starch, phosphorus exists as a natural ester of ' amylopectin ' and is carried along as an impurity throughout the esterification and purification. Its specific effect on the physical properties of the acetylated starches has not been fully demonstrated.

Methylcyclohexane may be used as an inert liquid for the determination of the specific gravity of the acetylated derivatives of starch.[132]

The acetyl derivatives of starch are usually characterised by determination of their (*a*) acetyl value, (*b*) behaviour in solution, (*c*) fusion temperature or melting point, (*d*) mechanical properties, (*e*) optical rotation, (*f*) solubility in various types of solvents, and (*g*) solubility and compatibility.[96]

Part A. Acetyl Value, Estimation of.—The estimation of acetyl value, by trans-etherification of an acetylated starch with methanol in the presence of *p*-toluenesulphonic acid, was used by Freudenberg and Harder in 1923.[38] By a modification of the Eberstadt method for the determination of acetyl in cellulose acetate, an acetyl derivative of starch is swollen with warm alcohol (75 per cent.) and then saponified, below 35° C., for 24 to 48 hours with aqueous alkali (0·25 *N*, initial concentration).[47] The excess alkali is back-titrated carefully with standard acid.[47]

By one procedure, 1 gm. of an acetyl derivative of starch is suspended in 50 ml. of carbon dioxide free water and titrated with 0·1 *N* sodium hydroxide solution until barely alkaline to phenolphthalein. This is a measure of the absorbed acid. One then adds

25 ml. of 0·5 *N* sodium hydroxide, heats the suspension while stirring, and boils the solution for five minutes. The excess base is then titrated with 0·5 *N* sulphuric acid and the acetyl value of the ester calculated from the amount of base required for the saponification.[19]

By another procedure, 1 gm. of the ester is placed in a glass-stoppered flask, covered with 10 ml. of methanol, allowed to stand for an hour, and then treated with 20 ml. of 1 *N* sodium hydroxide solution.[88] This mixture is allowed to stand at room temperature, with occasional agitation, for 12 hours and then titrated with 0·5 *N* hydrochloric acid. The amount of alkali required for the saponification is used to calculate the acetyl value.[88]

By the trans-etherification procedure, 0·2 to 0·3 gm. sample of the finely divided ester, previously dried *in vacuo* over phosphorus pentoxide, is placed in 20 ml. of anhydrous methanol in a reaction flask. Five to 10 ml. of 0·2 *N* sodium methoxide in methanol is then added slowly through a dropping funnel. The temperature is then raised to maintain the distillation of about 35 drops per minute into a receiver packed in ice. When the volume is reduced to about 5 ml., 20 ml. of anhydrous methanol is again added through the dropping funnel. Two additional portions of anhydrous methanol, 10 ml. each, are added and distilled. The distillate is then saponified and the excess alkali titrated.[141] A modification of the procedure makes use of *p*-toluenesulphonic acid as the trans-esterification agent.[23]

Part B. Behaviour of Acetyl Derivatives in Solution.—The procedures normally used in estimating the molecular weight of acetylated starches include: cryoscopic,[138] diffusion,[109] ebullioscopic,[138] osmotic pressure,[120] and viscosity measurements.[35]

Although high polymers frequently give abnormal results with respect to molecular weight determinations by the cryoscopic method,[123] this method has been used to some extent in the determination of the molecular weight of acetylated starches.[138] Even in the study of the derivatives of cellulose, the cryoscopic method may lead to erroneous results because of both the tendency of the solute to crystallise and the probable presence of impurities of low molecular weight.[39]

The diffusion measurements on acetyl derivatives of starch in solvents such as acetic acid and acetic anhydride indicate a molecular weight of about 225,000.[109] Ebullioscopic measurements for the estimation of the molecular weight of the esters of starch have proven unsuccessful.[138]

The osmotic pressure measurements on solutions of acetylated potato starches suggest a molecular weight of from 45,000 to as

much as 275,000 for the various fractions.[120] Comparable measurements on the triacetyl derivatives of 'amylose' and 'amylopectin' in tetrachloroethane give values respectively of 78,000 and 300,000.[82]

The comparative flow times of the acetic acid solutions of acetyl derivatives of starch have been determined.[31] The empirical formula,[119, 122]

$$\eta_{sp}/C = K_m M$$

relates the viscosity of a polymer in solution to its molecular weight. The specific viscosity, η_{sp}, is equal to the relative viscosity minus one. The C is the concentration of the solution in basal moles per litre. Km is a constant for a given homologous series in a specific solvent, and M is the molecular weight.[119] This formula is only relatively accurate.[84] The viscosity of a number of dilute solutions of acetyl derivatives of starch have been measured,[124] and is relatively independent of temperature.[120] The viscosity of a carefully prepared triacetyl ester of starch, in tetrachloroethane, however, is reported to vary appreciably with the temperature.[58] The viscosity of the triacetyl ester of 'amylose' is considerably higher than is that of the comparable ester of 'amylopectin'.[82] When the Kraemer intrinsicity viscosity[72] is plotted against concentration, for the triacetyl ester of potato starch, a straight line with a positive slope is obtained whereas the acetyl ester of cellulose gives a line with a negative slope.[88] The significance of viscosity measurements on starch pastes have been considered.[16]

The viscosity of aqueous solutions of acetylated starches have been measured by use of an Ostwald viscometer, at various concentrations, degrees of ageing, and temperatures.[20]

The viscosities were calculated from the equation $\eta = Adt - Bd/t$, where η is the viscosity in millipoise, d is the density of the solution, t is the time of flow in seconds, and A and B are constants peculiar to the viscometer. Some results are shown in Figs. 13 : 1 and 13 : 2.[20]

Part C. Fusion Temperature, Melting Point, and or Decomposition Temperature.—The fusion temperature, melting point, and or decomposition temperature of a number of acetyl derivatives of starch have been reported by different authors.[131] The usual method of determining melting points consists of recording three temperatures; that at which a pseudo-liquid or the first apparent melting occurs, that at which a semi-transparent stage begins to appear, and that at which the entire mass becomes transparent.[93] Further heating decomposes the sample.[20] In

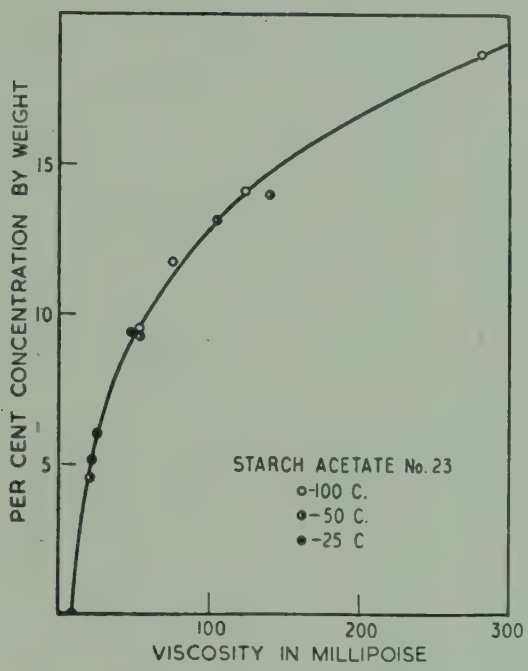

FIG. 13 : 1

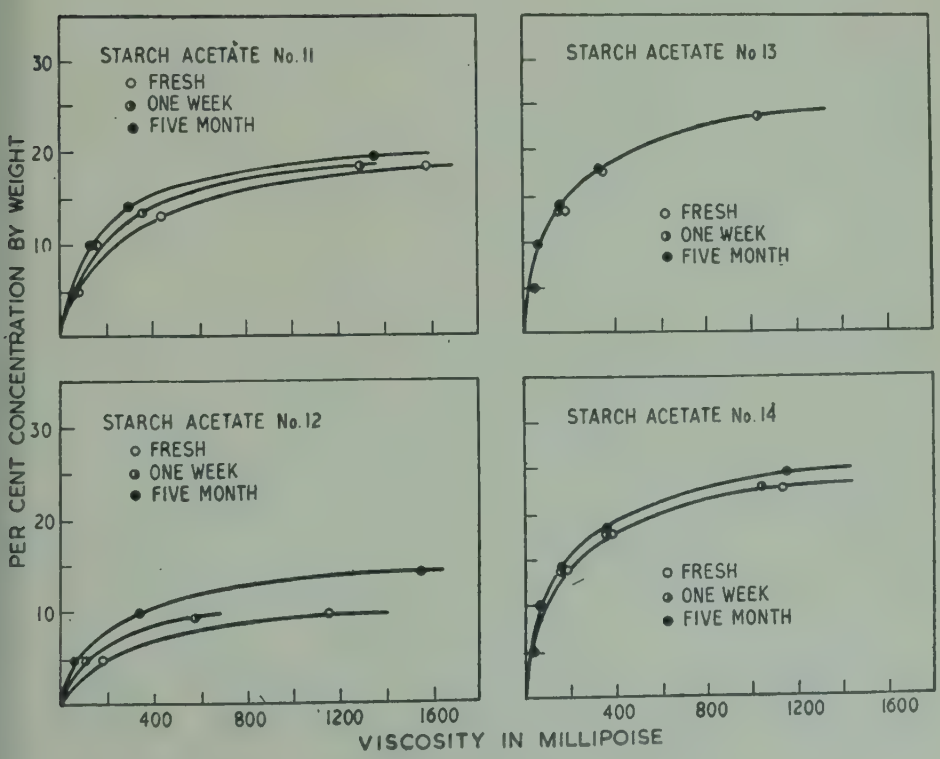

FIG. 13 : 2

TABLE XXV

Melting Points of Acetyl Derivatives[20]

Sample No.	Type of Ester	Free Acid per cent.	Acetyl Value per cent.	Ash per cent.	Melting Point: First Melting or decomposition	Melting Point: Transparent
11.	5-7%, Low Viscosity	0·30	6·60	0·26	240-264° C.	—
12.	5-7%, High Viscosity	0·36	4·90	0·03	235-268° C.	—
13.	9-11%, Low Viscosity	0·30	9·30	0·20	238-258° C.	—
14.	9-11%, High Viscosity	0·30	9·80	0·04	250-260° C.	—
15.	Triacetate, Low Viscosity	0·20	42·90	0·14	188° C.	205-212° C.
16.	Triacetate, High Viscosity	0·02	42·20	0·02	175° C.	220° C.
25.	Triacetate, Low Viscosity	0·02	41·00	—	181° C.	200-202° C.
26.	Triacetate, High Viscosity	0·02	41·60	—	180° C.	195-215° C.

the case of the water soluble esters, only a decomposition temperature is observed when the samples turn from a white to a brown.[20] Some results are recorded in Table XXV.[20]

It is apparent from these data that these products melt over a range of temperatures, extending over 14 to 35° C. or more. The best grades of triacetyl ester of starch are reported to melt, in sealed tubes, at 280 to 300° C.[96] These high fusion temperatures are another indication of the high degree of association of these molecules.

Part D. Mechanical Properties.—The triacetyl derivative of 'amylose' gives relatively strong films, whereas the corresponding ester of 'amylopectin' gives relatively weak films.[82] This indicates that good film-forming properties are dependent upon the presence of certain structural units of the string-type molecule. Acetyl derivatives of whole starch give moulded products which are brittle, clear, and transparent, whereas the comparable derivatives of 'amylose' may be moulded into strong and tough plastics.[89] The acetyl derivative of starch fraction A of Schoch,[110] forms films of high tensile strength and good extensibility, but the corresponding derivative of fraction B does not, although both give clear, transparent, and lustrous films.[140]

The X-ray analysis indicates that stretching produces orientation in films of acetylated 'amylose', but the effect is much less pronounced in comparable films of amylopectin.[143] The former are more linear than the latter and thus more susceptible to orientation by stretching.[143] The effect of plasticiser on stress-strain properties has been noted.[96]

Part E. Optical Rotation.—The specific rotation of different acetyl derivatives of starch in various solvents has been studied.[113] These measurements on the esters of starch, as well as those on raw starch, indicate that the β-type of linkage does not occur to any appreciable extent in the starch molecule.[83] Some data on optical activity of esters of starch are given in Table XXVI.[20]

Part F. Solubility.—The solubility of an esterified starch is dependent upon (1) the acetyl value, (2) the degree of degradation of the esterified molecule, (3) the degree of polymerisation of the ester, (4) the fractionation of the starch or the derivative, (5) the nature of the acetyl substituent, (6) the type of starch, and (7) the solvent and temperature.

Acetylated starches of low acetyl value (up to 25 per cent.) are reported to be water soluble.[26] If the derivatives are not degraded, however, it is likely that acetylated starches with acetyl values of 15 per cent. or above are not water soluble.[96] The

TABLE XXVI

Optical Rotation of Acetylated Starches

Acetyl ester of	$(a)_D$	0° C.	*Solvent used*	*Investigators*
Banana	+ 167°	20°	chloroform	Hawkins, Jones, Young[51]
Canna	164	—	chloroform	Hassid, Dore[49]
Corn	156·5	22	pyridine	Mullen, Pacsu[88]
Corn, 5-7 %, L.V.	225	20	water	Burkhard, Degering[20] No. 11
Corn, 5-7 %, H.V.	220·1	20	water	Burkhard, Degering[20] No. 12
Corn, 9-11 %, L.V.	188·6	20	water	Burkhard, Degering[20] No. 13
Corn, 9-11 %, H.V.	180·0	20	water	Burkhard, Degering[20] No. 14
Corn, Tri-, L.V.	166·7	20	chloroform	Burkhard, Degering[20] No. 15
Corn, Tri-, H.V.	172·5	20	chloroform	Burkhard, Degering[20] No. 26
Potato	177-9	18	chloroform	Baird, Haworth, Hirst[53]
Potato	168·4	20	chloroform	Brigl, Schinle[15]
Potato	158	22	pyridine	Mullen, Pacsu[88]
Potato	176·3	23	chloroform	Reich, Damansky[102]
Potato, amylopectin	170	19	chloroform	Hirst, Plant, Wilkinson[53]
Potato, amylose	170	19	chloroform	Hirst, Plant, Wilkinson[53]
Potato, amylose	172	20	chloroform	Freudenberg, Rapp[39]
Rice	162·4	19	chloroform	Brigl, Schinle[15]
Rice	171	20	chloroform	Hirst, Young[6]
Rice	157·9	22	pyridine	Mullen, Pacsu[88]
Tapioca	155	22	pyridine	Mullen, Pacsu[88]
Waxy corn	166	18	chloroform	Haworth, Hirst, Woolgar[54]
Wheat	151	22	pyridine	Mullen, Pacsu[88]

triacetyl derivatives of potato starch swell but are insoluble in acetone, benzene, chloroform, and ethyl acetate.[41, 58]

The degree of solvation of an esterified starch is exceptionally high in a polar solvent such as formic acid.[120] The 'amylose' molecules are less extended than are the cellulose molecules and are believed to be arranged in a spiral,[37] which accounts in part for their greater solubility. Acetylated 'amylose' is quite soluble in ethyl acetate.[82]

The triacetyl derivatives of starch are soluble, in general, with the exception perhaps of potato starch, in acetic acid, chloroethane, chloroform, tetrachloroethane, and other halogenated hydrocarbons. They are insoluble in alcohol, ethyl acetate, and, probably, in acetone and pyridine. The triacetyl ester of waxy corn starch, perhaps on account of its low molecular weight, is soluble in a variety of solvents.[96] A solution of an acetylated starch may be prevented from going mouldy by the use of a crystal of mercuric iodide.[66]

G. Solubility and Compatibility.—The solubility and compatibility of both cellulose acetate[34] and acetylated starches have been studied.[20] The solubility of triacetylated starches in plasticisers was determined at 25° C., 100° C., and 180° C., using 1 gm. of the ester to 10 gm. of the plasticiser.[20] The results are tabulated in Table XXVII.

The plasticiser retention of films cast from acetylated starches, in which L is a triacetylated starch of low viscosity and H is a comparable derivative of high viscosity, are given in Table XXVIII.

These data indicate that (1) naturally occurring vegetable oils do not dissolve triacetylated starches, (2) the low molecular esters are good solvents, (3) the high molecular esters are poor solvents, (4) liquids with high viscosities are poor solvents, (5) there is an appreciable loss in weight of the film on heating, (6) there is considerable loss in weight of the film on leaching, and (7) the films become brittle and opaque on leaching.

Section III. Other Acetyl and Aroyl Derivatives of Starch

The treatment of starch with formic acid at room temperature for 24 hours effects gelatinisation and the formation of a monoformate ester, which is stained red-brown by iodine.[70] Comparable results are obtained by heating the mixture, but the triformate is not obtained without continued treatment and consequent depolymerisation.[70]

The mono-, di-, and trichloroacetyl esters of starch are pre-

TABLE XXVII

Solubility and Compatibility of Triacetylated Starch with Plasticisers

Plasticiser	Solubility						Compatibility			
	25°C.		100°C.		180°C.		One Week		One Month	
	L	H	L	H	L	H	L	H	L	H
None	–	–	–	–	–	–	P	C	P	C
1. Dimethyl Phthalate	I	I	S	S	S	S	C	C	C	C
2. Diethyl Phthalate	I	I	S	S	S	S	C	C	C	C
3. Di-*n*-propyl Phthalate	I	I	I	I	S	S	P	C	P	C
4. Di-isopropyl Phthalate	I	I	I	I	S	S	P	C	P	P
5. Dibutyl Phthalate	I	I	I	I	S	S	P	N	P	N
6. Diamyl Phthalate	I	I	I	I	M	M	P	N	P	N
7. Dioctyl Phthalate	I	I	I	I	S	S	P	N	P	N
8. Dimethoxyethyl Phthalate	I	I	S	S	S	S	C	P	C	P
9. Diethoxyethyl Phthalate	I	I	S	S	S	S	P	C	P	C
10. Diphenyl Phthalate	I	I	I	I	S	S	P	N	P	N
11. Dibutoxyethyl Phthalate	I	I	I	I	S	S	N	C	N	C
12. Benzyl Phthalate	I	I	S	S	S	S	P	C	P	P
13. Dimethyl Sebacate	I	I	S	S	S	S	N	C	N	C
14. Diethyl Sebacate	I	I	I	I	S	S	N	N	N	N
15. Dibutyl Sebacate	I	I	I	I	S	S	N	N	N	N
16. Ditetrahydro Furfuryl Sebacate	I	I	I	I	S	S	N	P	N	P
17. Monotetrahydro Furfuryl Monobenzyl Sebacate	I	I	I	I	S	S	C	C	N	C
18. Dimethoxyethyl Adipate	I	I	S	S	S	S	C	P	C	P
19. Dicyclohexyl Adipate	I	I	I	I	S	S	P	N	P	N
20. Butyl Cellosolve Adipate	I	I	I	I	S	S	N	P	N	P
21. Methyl-*o*-Benzoyl Benzoate	I	I	I	I	S	S	P	C	P	C
22. Ethyl-*o*-Benzoyl Benzoate	I	I	I	S	S	S	C	C	C	C
23. Benzyl Benzoate	I	I	S	S	S	S	P	C	N	P
24. Diethyl Tartrate	I	I	S	S	S	S	C	C	C	P
25. Dibutyl Tartrate	I	I	S	S	S	S	C	C	C	C
26. Triacetin	S	S	S	S	S	S	P	C	P	C
27. Tripropionin	I	I	S	S	S	S	C	C	C	P
28. Tributyrin	I	I	S	S	S	S	P	P	N	P
29. Ethylene Glycol Diacetate	S	S	S	S	S	S	P	C	P	C
30. Triethylene Diacetate	S	S	S	S	S	S	C	C	C	P
31. Tetrahydro β-Naphthol Acetate	I	I	I	I	S	S	N	N	N	N
32. Pentaerythritol Tetraacetate	I	I	S	S	S	S	P	P	P	P
33. Glucose Pentaacetate	I	I	I	I	S	S	P	P	P	P
34. Sucrose Octaacetate	I	I	S	S	S	S	P	P	P	P
35. Ethylene Dipropionate	S	S	S	S	S	S	C	C	C	C
36. Diethylene Glycol Dipropionate	S	S	S	S	S	S	C	C	C	C
37. Ethylene Dibutyrate	I	I	S	S	S	S	C	P	C	P
38. Ethyl Succinate	S	S	S	S	S	S	C	C	C	P
39. Lauryl Salicylate	I	I	I	I	I	I	–	–	–	–
40. Cyclohexyl Laurate	I	I	I	I	M	M	N	N	N	N

TABLE XXVII (*continued*)

Plasticiser	*Solubility*						*Compatibility*			
	25°C.		100°C.		180°C.		*One Week*		*One Month*	
	L	H	L	H	L	H	L	H	L	H
41. Chlorinated Methyl Laurate	I	I	I	I	S	S	P	N	P	N
42. 2-Ethylhexyl Laurate	I	I	I	I	I	I	—	—	—	—
43. Ethyl Crotonate	I	I	I	I	S	S	P	P	P	P
44. Butyl Crotonate	I	I	I	I	S	S	P	P	P	P
45. Tributyl Citrate	I	I	S	S	S	S	P	C	P	C
46. Acetyl Triethyl Citrate	I	I	S	S	S	S	P	C	P	C
47. Acetyl Tributyl Citrate	I	I	I	I	M	M	N	N	N	N
48. Butyl Acetyl Ricinoleate	I	I	I	I	M	M	N	N	N	N
49. Butyl Stearate	I	I	I	I	M	M	N	N	N	N
50. Triethyl Phosphate	I	I	S	S	S	S	C	C	C	C
51. Tributyl Phosphate	I	I	S	S	S	S	P	C	P	C
52. Tricresyl Phosphate	I	I	I	I	S	S	P	N	P	N
53. Triphenyl Phosphate	I	I	S	S	S	S	P	C	P	C
54. Plasticiser 2 (Dow)	I	I	I	I	S	S	N	P	N	P
55. Plasticiser 5 (Dow)	I	I	I	I	S	S	P	P	P	P
56. Plasticiser 6 (Dow)	I	I	I	I	S	S	P	P	P	P
57. Plasticiser 7 (Dow)	I	I	I	I	S	S	N	P	N	P
58. Plasticiser 9 (Dow)	I	I	I	I	M	M	P	P	P	P
59. Santiciser 8	I	I	S	S	S	S	P	C	P	C
60. Santiciser 9	I	I	I	I	S	S	P	N	P	N
61. Santiciser 10	I	I	I	I	S	S	P	N	P	N
62. Santiciser B-16	I	I	I	I	S	S	P	P	P	P
63. Santiciser E-15	I	I	S	S	S	S	P	P	P	P
64. Santiciser M-17	I	I	S	S	S	S	P	C	P	C
65. Sanolite K	I	I	I	I	S	S	P	C	P	C
66. Sanolite MS	I	I	I	I	S	S	P	C	P	C
67. Sanolite MHP	I	I	I	I	S	S	P	C	P	C
68. Benzophenone	I	I	S	S	S	S	C	C	P	P
69. Acetamide	I	I	S	S	S	S	N	P	N	P
70. Acetanilide	I	I	I	I	S	S	P	C	P	C
71. Hercolyn	I	I	I	I	S	S	N	N	N	N
72. Abalyn	I	I	I	I	S	S	N	N	N	N
73. Sipalin AOM	I	I	I	I	S	S	P	N	P	N
74. Cyclonol	I	I	S	S	S	S	C	C	C	C
75. Triphenyl Guanidine	I	I	I	I	S	S	P	P	P	P
76. Camphor	I	I	I	I	S	S	P	C	P	P
77. N.N′-Diethyl-N. N′ Diphenyl Urea	I	I	S	S	S	S	P	C	P	C
78. Paraplex 5-B	I	I	I	I	S	S	N	N	N	N
79. Paraplex RG-2	I	I	I	I	S	S	P	N	P	N
80. Paraplex G-20	I	I	I	I	S	S	P	N	N	N
81. Flexol-Plasticiser 3-GH	I	I	I	I	S	S	P	C	P	C
82. Flexol-Plasticiser 3-GO	I	I	I	I	S	S	N	N	N	N
83. Butyl Acetal	I	I	I	I	I	I	—	—	—	—
84. Ethyl Acetanilide	I	I	S	S	S	S	P	C	P	P

TABLE XXVII (*continued*)

Plasticiser	*Solubility*						*Compatibility*			
	25°C.		100°C.		180°C.		*One Week*		*One Month*	
	L	H	L	H	L	H	L	H	L	H
85. Ethyl-*p*-Toluenesulphonate	I	I	S	S	S	S	P	P	P	P
86. Diphenyl Ether	I	I	I	I	M	M	P	C	P	N
87. Polychlor Naphthalene 93°	I	I	I	I	I	I	–	–	–	–
88. Polychlor Naphthalene 123°	I	I	I	I	I	I	–	–	–	–
89. Polychlor Naphthalene 135°	I	I	I	I	I	I	–	–	–	–
90. Plasticiser E-30	I	I	I	I	S	S	N	N	N	N
91. Plasticiser E-40	I	I	I	I	S	S	N	N	N	N
92. Plasticiser E-50	I	I	I	I	S	S	N	N	N	N
93. Plasticiser E-60	I	I	I	I	S	S	P	N	P	N
94. Aroclor 1242	I	I	I	I	S	S	P	P	P	P
95. Aroclor 1248	I	I	I	I	S	S	P	C	P	P
96. Aroclor 1254	I	I	I	I	S	S	P	P	P	P
97. Aroclor 1260	I	I	I	I	S	S	N	N	N	N
98. Soybean Oil	I	I	I	I	I	I	–	–	–	–
99. Linseed Oil	I	I	I	I	I	I	–	–	–	–
100. Cottonseed Oil	I	I	I	I	I	I	–	–	–	–
101. Castor Oil	I	I	I	I	I	I	–	–	–	–
102. Soyapole 75	I	I	I	I	I	I	–	–	–	–
103. Ditetrahydro Furfuryl Maleate	I	I	S	S	S	S	C	C	C	C
104. Carbowax 1500	I	I	M	M	S	S	P	C	P	C
105. Carbowax 4000	I	I	I	I	M	M	N	N	N	N

S—Soluble; M—Partially soluble; I—Insoluble; C—Compatible; P—Compatible, but cracked; N—Non-compatible; H—High Viscosity Starch Triacetate; L—Low Viscosity Starch Triacetate.

TABLE XXVIII

Plasticiser Retention by Starch Acetate Films

Plasticiser	*Ester*	*Original Plasticiser Content* %	*Loss in Heating* %	*Loss on Leaching* %
1. None	L	00·00	5·38	14·96
2. None	H	00·00	6·89	13·45
3. Dimethyl phthalate	L	10·60	9·80	11·95
4. Dimethyl phthalate	H	8·64	7·72	14·09
5. Diethyl phthalate	L	9·46	5·16	11·62
6. Diethyl phthalate	H	8·85	7·82	13·12
7. Di-*n*-propyl phthalate	L	9·67	5·53	10·04
8. Di-*n*-propyl phthalate	H	7·82	5·24	10·16
9. Diisopropyl phthalate	L	9·62	4·43	12·63
10. Diisopropyl phthalate	H	8·52	4·24	9·66
11. Dibutyl phthalate	L	7·64	4·30	11·20

TABLE XXVIII (*continued*)

Plasticiser	*Ester*	*Original Plasticiser Content* %	*Loss in Heating* %	*Loss on Leaching* %
12. Dibutyl phthalate	H	12·98	9·97	10·32
13. Diamyl phthalate	L	11·62	5·46	10·28
14. Diamyl phthalate	H	12·11	5·94	8·21
15. Diphenyl phthalate	L	9·48	5·59	11·68
16. Diphenyl phthalate	H	8·89	6·67	9·78
17. Dibenzyl phthalate	L	10·79	6·57	9·65
18. Dibenzyl phthalate	H	8·82	5·51	10·28
19. Dimethoxyethyl phthalate	L	11·32	7·20	12·52
20. Dimethoxyethyl phthalate	H	9·78	6·84	12·02
21. Diethoxyethyl phthalate	L	9·85	7·21	10·71
22. Diethoxyethyl phthalate	H	11·90	8·02	10·26
23. Dibutoxyethyl phthalate	L	11·53	3·59	9·35
24. Dibutoxyethyl phthalate	H	12·18	4·47	8·88
25. Triethyl phosphate	L	9·39	6·20	14·03
26. Triethyl phosphate	H	9·35	8·00	15·19
27. Triphenyl phosphate	L	8·74	4·21	10·52
28. Triphenyl phosphate	H	10·74	5·32	11·53
29. Tributyl phosphate	L	17·79	3·50	5·44
30. Tributyl phosphate	H	11·38	8·11	9·12
31. Tricresyl phosphate	L	10·95	4·18	11·90
32. Tricresyl phosphate	H	10·22	3·62	8·52
33. Tributyl citrate	L	8·61	4·03	9·21
34. Tributyl citrate	H	11·99	5·23	8·23
35. Acetyl triethyl citrate	L	9·84	4·62	9·65
36. Acetyl triethyl citrate	H	8·70	4·04	9·37
37. Dibutyl tartrate	L	9·44	3·88	11·20
38. Dibutyl tartrate	H	8·62	7·27	15·40
39. Diethyl tartrate	L	10·63	3·62	17·81
40. Diethyl tartrate	H	10·93	7·14	11·35
41. Methyl-*o*-benzyl benzoate	L	9·41	6·85	15·58
42. Methyl-*o*-benzyl benzoate	H	10·55	6·38	14·48
43. Ethyl-*o*-benzyl benzoate	L	9·03	4·24	14·17
44. Ethyl-*o*-benzyl benzoate	H	9·59	12·02	13·88
45. Triphenyl guanidine	L	10·18	3·09	12·24
46. Triphenyl guanidine	H	10·79	6·22	11·98
47. Triacetin	L	12·01	7·03	17·50
48. Triacetin	H	11·72	6·29	21·40

pared by heating starch either with the appropriate acid or with the acid anhydride in the presence of sulphuric acid.[69] The trichloroacetyl derivatives of starch may be prepared also by treating the triacetyl derivative of starch with phosphorus pentachloride at 135-140° C.[106]

The glyoxylic ester of starch was prepared in 1895 by the treatment of starch with glyoxylic acid.[14] It is obtained also when starch is treated with chloroacetic acid in the presence of aqueous sodium hydroxide.[21]

A mixture of the propionyl derivative of starch is prepared by refluxing starch with propionic acid and propionic anhydride.[79] The lower derivatives are water soluble whereas the higher acylated starches are soluble in organic solvents. The triester may be obtained also by treating pyridine-treated starch with propionic anhydride and distilling the water as a pyridine-water azeotrope.[79] A mixture of the butyryl derivative of starch, mostly the triester, is obtained by treating pyridine-treated starch with butyric anhydride.[88] The triester is soluble in organic solvents.[88] The tripropionyl and the tributyryl derivatives of starch form brittle, clear plastics, which are somewhat softer than are those of the triacetyl derivatives.[89] Similar results have been noted for the cellulose esters, which confirms the belief that the attraction between the starch molecules is rendered less effective by larger aliphatic substituents.[89]

A mixture of the stearoyl esters of starch may be prepared by treating gelatinised starch with stearic anhydride.[78] Starch esters of lauric, myristic, and palmitic acids may be prepared similarly. Typical procedures are:

The dilauryl ester of starch is prepared by heating 1 part of dry starch for 2 hours at 100° C. with 5 parts of lauroyl chloride in 2·5 parts of pyridine and 3 parts of toluene.[44] The derivative is insoluble in acetone, alcohol, and water, but is soluble in benzene, chloroform, and tetrachloroethane. A benzene-chloroform solution of this ester, on evaporation, forms a brittle film.[44]

The tripalmityl ester of starch is prepared by heating 1 part of starch with 6 parts of palmitoyl chloride in quinoline at reflux temperature for about 3 hours.[9] The quinoline may be replaced by 4 parts of benzene and 1·8 parts of pyridine, the mixture refluxed for 30 minutes, and the partially esterified product precipitated with ethanol.[9]

The esters and mixed esters may be prepared by the use of the appropriate anhydride or mixture of anhydrides on pretreated starch[46]. The film properties are improved with the use of increasing amounts of acetic anhydride.[46]

The higher aliphatic esters of starch are usually prepared by treating starch with either the appropriate acyl halide or anhydride in the presence of alkali or a tertiary organic base.[10] Organic bases which have been used include dimethylaniline,[105] picoline,[76] pyridine,[8] and quinoline.[65]

A starch hexapalmitate and a starch hexastearate have been prepared and characterised,[65] as has also an oleic-sulphuric acid derivative of both starch and dextrin.[74]

A benzoyl-lauroyl ester of starch may be prepared by mixing 1 part of starch with 1 part of 40 per cent. sodium hydroxide solution, refluxing the mixture in benzene, and adding dropwise a mixture of benzoyl and lauroyl chlorides. Heating should be continued a few minutes after the last of the acyl chlorides is added.

A mixture of benzoyl esters of starch is prepared by heating starch in pyridine with benzoyl chloride for 72 hours at 75° C.[104] The triester is insoluble in alcohol and ether but is soluble in acetone, chloroform, and pyridine.[104] The tribenzoyl ester may be produced also by allowing pyridine-treated starch to stand in benzoyl chloride at room temperature for about 12 days.[56] The optical rotation of the triester in chloroform is $[a]_D^{22} + 80°$.[56] This ester was prepared in 1893 by treating soluble starch with alkali and then with benzoyl chloride.[24]

The di- and tricinnamyl derivative of starch is prepared by heating dry starch in pyridine with cinnamyl chloride at 75° C.[103]

The tritosyl starch is prepared by treating a pyridine-treated starch with tosyl chloride (*p*-toluene-sulphonyl chloride) in the presence of pyridine at room temperature for about 9 days.[56] This derivative decomposes at 235° C., is insoluble in most solvents, and yields 2,3-ditosyl-6-iodostarch when treated with sodium iodide in acetone for 3 days at 100° C.[56] These derivatives, when treated with hydrogen bromide in glacial acetic acid, yield respectively 1-α-bromo-2, 3, 6-tritosyl-4-acetylglucose and 1-α-bromo-2, 3-ditosyl-4-acetyl-6-iodoglucose.[55]

The 3, 4-dichlorobenzenesulphonyl ester and similar derivatives of starch may be prepared by treating dry starch with the appropriate aroyl chloride in the presence of an alkali or tertiary amine base.[60]

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Chapter 14

THE ETHERS OF STARCH

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The first recorded interest in the ethers of starch is disclosed in a patent, which was issued in 1912.[19] Part of the work since then on the production of ethers of starch has been motivated entirely by structural considerations of the starch molecule.[8]

Alkylating agents which have been used in the synthesis of ethers of starch include: alkyl halides,[20] allyl bromide,[26] allyl chloride, benzyl chloride,[10] chlorotriphenylmethane,[16] diazomethane,[25] epichlorohydrin,[4] ethylene chloride,[4] ethylene chlorohydrin,[4] ethylene oxide,[2] lauryl chloride,[9] methyl sulphate,[17] *p*-nitrobenzyl chloride,[23] and sodium alkyl sulphates.[5]

Condensing agents which have been reported for the alkylation of starch include: aqueous alkali,[21] 30 per cent. aqueous sodium hydroxide,[15] calcium chloride,[6] pyridine,[16] pyridine in toluene,[9] and a tertiary amine organic base.[21]

Consideration has been given to both the raw material and the procedure for the production of ethers of starch. Some of the ethers have been prepared and characterised.[27] Patents cover the production of the ethers of starch by treatment of starch with alkyl halides in the presence of an appropriate condensing agent.[20] The treatment of starch with sodium alkyl sulphates under proper conditions results in the production of the corresponding ethers of starch.[5] A variation in the experimental conditions results in a variation in the properties of the product of the reaction.[5]

The patent literature covers the production of various ethers by use of an alkylating agent in caustic.[7] Directions for the preparation of the methyl derivatives of starch are given by Karrer[18] and others,[11] and those for the synthesis of the ethyl ethers of starch are described rather fully by Lilienfeld.[19]

I. Preparation of Ethers of Starch. The methylated starches have been prepared from both raw starch[17] and triacetyl starch.[18] Raw starch of both rice and potato, after 24 successive methylations with methyl sulphate and caustic give only a 25 per cent. yield of trimethyl starch.[17] A (1) dimethylstarch[18] and a (2)

trimethylstarch[15] may be obtained in satisfactory yields by the following procedures :

(*a*) *Dimethyl starch from triacetyl starch.*—Powdered triacetyl starch (20 gm.) is treated with 30 per cent. sodium hydroxide solution (224 ml.) and methyl sulphate (80 ml.), both added gradually and simultaneously with vigorous mechanical stirring.[15] The temperature is maintained at 50° C. for 75 minutes, then at 70° C. for another 75 minutes, and finally at 100° C. for 30 minutes. Mild alkalinity is maintained in the reaction flask throughout. The solution is then cooled, neutralised, filtered, and the solid residue extracted with boiling chloroform to give about an 80 per cent. yield of dimethyl starch.[15] The triacetyl starch was prepared from potato starch by use of acetic acid and acetic anhydride in the presence of chlorine and sulphur dioxide.[15]

(*b*) *Trimethyl starch from triacetyl starch.*—Powdered triacetyl starch (25 gm.) is dissolved in acetone (250 ml.) and treated, simultaneously and gradually, with methyl sulphate (100 ml.) and 30 per cent. sodium hydroxide solution (280 ml.), while the reaction mixture is maintained at 56° C.[15] The acetone is then distilled, the mixture maintained at 100° C. for 30 minutes, cooled, neutralised, and filtered. Both the filtrate and the residue are extracted with chloroform and the chloroform recovered to give a 96 per cent. yield of trimethyl starch.[15]

Similarly, the acetyl dextrins may be converted into methyl dextrins by dissolving in acetone and treating, slowly and simultaneously, with methyl sulphate and 30 per cent. aqueous sodium hydroxide at about 53° C.[13]

The acetyl derivative of *α-amylodextrin*, when dissolved in acetone and then treated, slowly and simultaneously, with methyl sulphate and 30 per cent. aqueous sodium hydroxide at 55° C., yields the methyl α-amylodextrin.[14] The acetyl derivative of amylose may be deacetylated and methylated simultaneously by use of methyl sulphate and 30 per cent. aqueous sodium hydroxide in acetone at 55° C.[1]

The acetyl derivative of *α-amylodextrin* (prepared by treating α-amylodextrin with acetic anhydride in pyridine) may be converted to the methyl ether also by solution in acetone and treatment with methyl sulphate and 30 per cent. aqueous sodium hydroxide, slowly and simultaneously, at room temperature for 5 hours.[12]

A glycollic acid derivative of starch has been used also for the preparation of the corresponding methyl ether.[3]

The ethyl ether of starch may be prepared by effecting ethylation of starch with ethyl sulphate in the presence of alkali.[24a]

One hundred grammes of starch (10 per cent. H_2O) is treated with 900 ml. of 10 per cent. sodium hydroxide solution in a large alkylation apparatus and then 200 ml. of 30 per cent. sodium hydroxide solution is added slowly with stirring. The mixture is heated to 60° C., 200 ml. of ethyl sulphate added dropwise with stirring, and the mixture then heated to 100° C. for an hour. The mixture is cooled, divided for convenience into two portions, and 300 gm. of finely divided sodium hydroxide added to each portion. Each portion is then warmed up to 60° C. and treated over a period of 2 hours with 300 ml. of ethyl sulphate. The solutions are finally heated on a water bath at 100° C. for an hour, neutralised and filtered or filtered 'as is', then washed well and dried. The resulting product is soluble in organic solvents but insoluble in water (yield, 27·65 per cent., 2·13 ethoxy groups per anhydroglucose unit).[24a]

The patent literature discloses the propyl ether of starch,[28] which may be synthesised by heating together in a stirred or rocking autoclave, at 140° C. for 24 hours, 30 gm. of corn starch (10 to 15 per cent. H_2O), 60 gm. of solid sodium hydroxide, 450 gm. of a 40 per cent. solution of sodium hydroxide, and 277 gm. of *n*-propyl chloride.[24] The reaction mixture is subjected to steam distillation to remove the lower alcohols and lower ethers.[24] The grey, solid residue is extracted with glacial acetic acid, filtered, and the filtrate poured, with efficient agitation, into an excess of cold water. The white product which floats to the surface is filtered, washed with sodium carbonate, washed with distilled water, and dried (yield, 30 gm.).[24]

The butyl ether of starch is prepared by heating, in a rocking or stirring type autoclave at 165° C. for 24 hours, 15 gm. of corn starch, 250 gm. of 40 per cent. sodium hydroxide, and 160 gm. of *n*-butyl chloride.[24] The excess butyl chloride, the butyl alcohol, and the butyl ether are then removed by steam distillation.[24] Upon cooling, a compact, brittle solid is obtained. This is dissolved in glacial acetic acid and the solution poured into an excess of cold water to precipitate the ether. This purification step may be repeated. A white, fluffy product (18·4 gm.) is obtained, which is soluble in some organic solvents but insoluble in water.[24]

The treatment of starch with lauryl chloride in the presence of pyridine and toluene at 100° C. for 2 hours and subsequent precipitation with alcohol, gives a dilauryl derivative (180 per cent. yield).[9] This ether is soluble in benzene and in chloroform and is non-inflammable.[9]

The treatment of starch with chlorotriphenylmethane in pyri-

dine yields the corresponding ether, which swells in organic solvents and is readily decomposed by water to give starch and hydroxytriphenylmethane.[16]

The hydroxy derivatives of starch are prepared readily by the reaction between starch and alkene oxides, particularly ethylene oxide, in the presence of aqueous alkali.[2] A different procedure condenses starch with chemicals such as ethylene chloride, ethylene chlorohydrin, and epichlorohydrin, in the presence of appropriate condensing agents, to yield hydroxy ethers of starch.[4]

The mono allyl ether of both corn and potato starch is prepared by treatment of the respective starches with allyl bromide in the presence of aqueous alkali.[26] Allyl starch is now produced commercially by condensing allyl chloride with starch.

The benzyl derivatives of starch are prepared from different starches by treatment of the starch under study with benzyl chloride in the presence of sodium hydroxide.[10] These derivatives have colloidal and paste properties which indicate certain commercial uses.[10] Benzylated starches vary with the source of the starch. These ethers are soluble in acetone, alcohol, and the aromatic hydrocarbons.[22] The films and castings are brittle and glossy.[22]

The treatment of alkali starch with compounds such as *p*-nitrobenzyl chloride yields derivatives which in turn may be reduced and diazotised and coupled to other compounds.[23]

II. Properties of Ethers of Starch.[24]—The methyl ether of starch, like the comparable derivative of cellulose, is water soluble.[24] If the ethyl ether contains only one ethoxy group per anhydroglucose unit, the product is water soluble, whereas if the product contains two or more ethoxy groups per anhydroglucose unit, it is insoluble in water but soluble in organic solvents.[24] The propyl and butyl ethers are usually insoluble in water but soluble in certain organic solvents.[24]

The ethyl, propyl, and butyl ethers of starch are all soluble in acetone, amyl acetate, butanol, and toluene.[24] The ethyl ether is soluble in 95 per cent. ethanol but insoluble in petroleum ether. The propyl derivative is soluble in 95 per cent. ethanol and in tetrachloroethane and partially soluble in petroleum ether. The butyl ether is soluble in tetrachloroethane, but only partially soluble in ethanol and petroleum ether.[24]

These alkyl ethers of starch are compatible with commonly used plasticisers such as dibutyl phthalate, ethox, flexol, hercolyn, tributyl citrate, and tributyl phosphate.[24]

The shrinking or softening point of the ethyl, propyl, and butyl ethers of starch described herewith are, respectively, 140-145° C

105-110° C., and 73-76° C.[21] The corresponding specific gravities at 27° C. are 1·14, 1·05, and 0·88. This indicates that a decrease in specific gravity results from an increase in the carbon content of the alkyl substituent. These products, like the cellulose ethers, have a large spreading power in comparison with that of more dense materials such as the acetates or nitrates of cellulose or starch.[24]

The viscosities of these alkylated products vary inversely as the chain length of the alkyl substituent.[24] The viscosities, expressed in centipoises are: ethyl, 2·92, propyl, 1·97; and butyl, 1·06. The relative flow times in seconds are: ethyl, 586; propyl, 390; and butyl, 210.[24]

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Chapter 15

THE INORGANIC DERIVATIVES OF STARCH

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I. The Halogen Derivatives of Starch.—The first recorded treatment of starch with halogen is that of an observation by Liebig in 1829 that chlorine or chlorous acid affect starch.[31] Some 78 years later Moissan observed that starch is attacked by fluorine with the deposition of carbon.[34]

In 1929 Bergmann and Ludewig presented evidence which indicated that bromine in the presence of potassium bromide and iodine in the presence of potassium iodide undergo reaction with certain starches or starch derivatives in some manner which involves a chemical union.[4] They attributed the reaction to the presence of the oxygen-bridge atoms in the starch molecule.[4] (See p. 239.)

More recently Barham and Thompson have studied the liquid phase chlorination of both raw starch and of pretreated starch.[52] The reaction is carried out in an autoclave at relatively low temperatures. The essential feature for successful chlorination is the maintenance of the hydrogen chloride concentration at a low value throughout the initial stages of the reaction either through its removal during the reaction or through its deactivation by the use of a tenfold excess of liquid chlorine. Failure to observe these precautions leads to destructive side-reactions and subsequent carbonisation of the starch. The products obtained comprise a series of partly-oxidised, partly-substituted derivatives containing up to 58 per cent. chlorine and possessing from 1 to 6 chlorine atoms per glucose residue.

The reaction is characterised by a prominent induction period of from 8 to 12 hours, at 70° C., at the conclusion of which the monochloride is rapidly formed. In a sequence of consecutive reactions the dichloride and the trichloride are likewise formed at 70° C., although the formation of the trichloride requires careful disorganisation of the starch. The dichloride is normally obtained in 36 hours at 70° C. (29·66 gm. vacuum oven dried maize starch, 525 gm. chlorine).

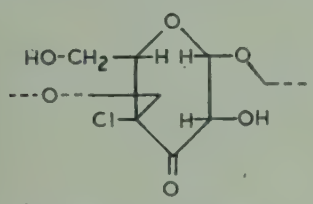
The trichloro derivative is prepared by treating 10 gm. of oven-dried starch (0·38 per cent. water) in an autoclave with 580 gm. of chlorine for seven 24-hour periods at 30, 40, 50, 55, 59·5, 62 and 66° C. respectively, and finally a 24-hour treatment at 70° C. to give a yield of about 13·12 gm.

The dichloride and the trichloride are homogeneous, undegraded, lemon-yellow granular products containing 31·24 and 41 per cent. chlorine respectively. The monochloride is a yellowish, nearly white, granular product of 18·22 per cent. chlorine.

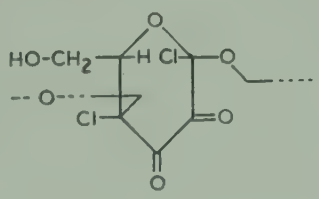
A tetrachloro derivative, containing 48 per cent. chlorine, is obtained directly from the dichloro derivative by withdrawing the vapours from the autoclave and then recharging with chlorine and heating at 100° C. for 24 hours.

Similarly, a hexachloro derivative, containing 58 per cent. chlorine, is obtained from the tetrachloride by further chlorination with a fresh charge of chlorine for 24 hours at 123° C. Both the tetrachloride and the hexachloride show evidence of degradation, undoubtedly due to the use of higher temperatures, and as a result are low melting solids which are soluble in liquid chlorine.

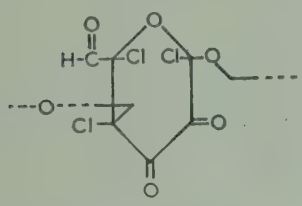
The structural units involved in the successive stages as postulated by Barham and Thomson, are :



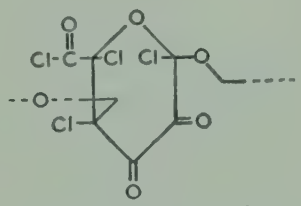
Monochloro Derivative



Dichloro Derivative



Trichloro Derivative



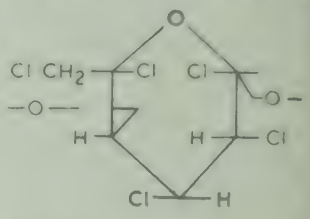
Tetrachloro Derivative

The use of phosphorus pentachloride for the synthesis halogen derivatives of starch has been studied by Barham, Stic[illegible] No. and Caldwell, etc.

An intimate mixture of starch and finely powdered phos pentachloride react, under anhydrous conditions at atm[illegible], 455. pressure, to give chlorine derivatives of starch (60 to 5[illegible] Cl).[2] About 10 equivalents of phosphorus pentachlori[illegible]*a. Acta.*, 1940, per glucose unit at an initial temperature of 100 to 1[illegible]

15 hours the temperature is increased to 125 to 135° C. and finally raised to 160 to 170° C. The suggested equation is :

$$C_6H_7O_2(OH)_3 + 5\ PCl_5 \rightarrow C_6H_5O_2Cl_5 + 5\ HCl + 3\ POCl_3 + 2\ PCl_3.$$



The proposed structure of the substituted glucose unit, accordingly, is believed to be that shown at the right.[2]

A typical procedure is to grind together, under anhydrous conditions, 40 gm. of dry starch (0·247 equivalent mole of anhydro-glucose) and 540 gm. (2·59 moles) of phosphorus pentachloride.[2] The mixture is then heated, at atmospheric pressure under anhydrous conditions, at 100 to 105° C. for 15 to 17 hours ; then at 125 to 135° C. for 24 or more hours ; and, finally, at 160 to 170° C. for 8 hours or more.[2] The product is then thoroughly washed with ice water, filtered, and dried (60 to 63 per cent. Cl).[2]

This derivative is relatively stable and is believed to be a 1, 2, 3, 5, 6-pentachlorostarch.[2]

II. The Nitric Acid Esters of Starch.—The nitric acid esters of starch were first produced in 1833 and then did a Rip Van Winkle for almost a century before commercial production began on a relatively large scale. The nitrates of starch are excellent explosives and are used extensively in blasting compositions for certain types of mining and for quarrying. Prior to World War II, the principal starch used in the production of these esters in the United States was tapioca. Since then corn starch has been used successfully.

The first recorded nitric acid derivative of starch is xyloidine, which was prepared in 1833 by treating potato starch with cold, concentric nitric acid. The use of this product in the treatment of textiles, it was noticed, imparted a stiffness and impermeability even after treatment with boiling water.[12] Eleven years later (1844) appeared a detailed procedure for the production of xyloidine and a theoretical consideration of the constitution of this derivative.[1] Subsequently (1846) a critical study of xyloidine and pyroxylin indicates that they differ with respect to both chemical composition and physical properties.[39] The utilisation of monoidine as a military explosive was considered.[39] reactive transformations which accompany the treatment of starch at 70° Mixed acid were studied (1847) by observing the progress of disorganization on a slide under a microscope.[29] It was observed obtained moreover, that the starch granule is composed of an inner starch, 525 gr portion, and that the inner portion gives a blue color- odine whereas the outer portion does not.[38]

Sixteen years after the first preparation of xyloidine, a detailed report (available at the Columbia College of Pharmacy) was published (1849) on the preparation and properties of starch nitrate.[42] Four years later (1853) the nitrate of starch was decomposed by treatment with an aqueous solution of ferrous salts to regenerate the starch.[3] In 1860, the nitric acid ester of dextrin (a degraded starch) was prepared.[3]

Two nitric acid esters of starch were prepared in 1862.[3] The one (6·7 per cent. N) is soluble in aqueous acetic acid but insoluble in the usual organic solvents, whereas the other (11 per cent. N) is insoluble in water but soluble in acetone, alcohol, and ether. Both of these compounds are optically active and they are both decomposed by aqueous solutions of ferrous salts to regenerate the starch and liberate nitric oxide.[3]

The heat exchange of the reaction of mixed acid on starch to give xyloidine was studied in 1876.[5] In 1892 a review was published, which covered the history of the preparation and properties of the nitric acid esters of starch.[35] Procedures were included for the production of esters of 10·5 to 13·5 per cent. nitrogen content, and evidence was cited to support the belief that these derivatives are true esters and not nitro compounds.[35]

Two methods reported in 1898 for the nitration of starch yield products which contain from 13·9 to 14·04 per cent. nitrogen.[54] Both derivatives are soluble in alcohol and ethyl acetate, both are comparatively stable at 50° C., but both decompose violently at 100° C.[54] They were considered to be the hexanitrates of starch.[54]

The determination (1899) of the molecular weights of the nitrates of starch (7·8 to 11·5 per cent. nitrogen), by the lowering of the freezing point of acetic acid, gave a value of about 987.[13] The treatment of these esters with ammonium sulphide solution, resulted in the production of soluble starch.[13] Subsequent determinations on the molecular weight of nitrates of starch (13·4 per cent. nitrogen) by the ebulliometr method gave a value of 1,845.[48]

The nitric acid ester of starch was decomposed (1908) by treat ment with alcoholic sodium hydroxide, the resulting mixt acidified and treated with a hydrazine, and an osazone isol similar to that obtained from oxypyruvic acid.[10]

Four varieties of starch were nitrated (1910) with mix to give esters which contain from 12·86 to 13·85 r nitrogen and which are soluble in alcohol and eth viscosity of a 5 per cent. solution of these products v to be only one nine-thousandth of that of a compa

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of the nitrate of cellulose.[6] A comparative study was made (1917) for the determination of nitrogen in the nitrates of starch by the use of the nitrometer and the nitro method.[18] A relatively complete and concise review of the earlier work on the nitrates of starch was published in 1922.[28]

(*a*) *Preparation of Nitric Acid Esters of Starch.*—The nitration of starch was studied in 1935 by Berl and Kunze.[9] Mixed acid gives a better product than does concentrated nitric acid, and a nitric-phosphoric acid mixture gives better results than does mixed acid.[9] The phosphoric acid in the latter case becomes fixed in the nitric acid ester to the extent of about 0·19 per cent. or more. With mixed acid small amounts of sulphuric acid also become fixed.

The nitrogen content of these esters is 13·0 ± 0·1 per cent. as compared to a theoretical value of 14·14 per cent.[9] The values in the literature of 14·10 per cent. or thereabout may result from (*a*) incorrect analytical methods, (*b*) appreciable degradation of the nitrated ester, or (*c*) careful fractionation of the product.

No apparent reaction accompanies the treatment of dry starch with 100 per cent. phosphoric acid at 0° C.[9] The addition of ice-cold nitric acid to the mixture is accompanied by rapid nitration, the nitrogen content of the product reaching a maximum in about 10 minutes.[9] The product is very stable and has a viscosity much higher than that of the derivative obtained by the nitration of starch with either mixed acid or nitric acid.[9]

A comparative study of osmotic pressure measurements of solutions of nitrates of starch, produced by the nitric-phosphoric acid mixture versus those produced by the use of mixed acid, indicate that degradation in the former case amounts to about 36 to 46 per cent. whereas in the latter case it reaches values of 40 to 70 per cent.[49]

Caesar and Goldfrank dry starch to a moisture content of less than 1 per cent., suspend the dry starch in an inert solvent such as chloroform or carbon tetrachloride, and then nitrate with nitrogen pentoxide, in the presence of a nitric acid binding agent such as sodium fluoride, at a temperature of 16 to 18° C.[15] The of reaction mixture is filtered, washed with chloroform, slurried mom cold water, filtered, washed with hot water, and stabilised reactioiling with dilute ammonium hydroxide.[15] The product at 70° (12·14 per cent. N) is stored in distilled water or 50 per cent. disorganit[5]

obtained i extraction has been used to separate the nitric acid esters arch, 525 gr fractions, which differ appreciably in their nitrogen their physical properties.[41]

(*b*) *Properties of Nitric Acid Esters of Starch.*—A critical comparison of the nitrate esters of starch with the native starch indicates the presence of at least two types of starch and two types of esters, the 'amylopectin nitrate' and the 'amylose nitrate'.[8] These esters are separated by grinding up the aqueous suspension and permitting fractional sedimentation.[8] The 'amylose nitrate' is denser, has a higher nitrogen content, and dissolves in acetone to give a clear solution, whereas the 'amylopectin nitrate' tends to form a slimy suspension in acetone.[8]

The nitrates of starch are usually relatively unstable, but the derivative obtained from the use of a nitric-phosphoric acid mixture, or nitrogen pentoxide-chloroform-sodium fluoride mixture, on dry starch is much stabler than is that obtained from the use of nitric acid or mixed acid on starch. Since the relative stability of the nitrates of starch appears to be a function of their preparation and purification, it is likely that their decomposition is initiated to some extent at least by traces of sulphate or other impurities.

The relative stability of the nitrates of starch may be increased by (*a*) the use of purified starch, (*b*) thoroughly washing with hot water to remove traces of acid, and/or (*c*) treatment with aniline,[36]

TABLE XXIX

Effect of Precooking under Pressure on Viscosity

Temperature	*Pressure*	*Viscosity of nitrate*
°C.	atmospheres	poises
90	0·715	48,000
100	1	38,000
110	1·4	37,140
120	2·0	34,760
130	2·7	28,100
150	4·7	1,600
160	6·1	42
170	7·8	14
180	10·0	6

t.,
ROP,

alkaline solutions,[36] carbonates,[43] dilute ammonium hydro 2, 130.
or methanol.[7]

If solutions of the nitrates of starch are treated with
solutions of alkalis, ammonium hydrogen sulphide, See also No.
sulphate solution, denitration occurs with the fc
soluble starch.[14] This is indicative that either the 1886, 7, 455.
cedure, or the denitration reaction, or both caus 41.
tion of the starch molecule.

The viscosity, also, of the nitrates of sta *Helv. Chem. Acta.*, 1940,
function of the type of starch and the m

The viscosity of a solution of nitrates of starch, with identical nitrogen content, is dependent in part at least upon (*a*) the composition and type of the nitrating agent, (*b*) the condition and type of the starch, (*c*) the time of nitration, (*d*) the temperature of nitration, and (*e*) certain other variables.

One of the variables is pretreatment. The viscosity of the nitrate derivative of a pretreated starch is less, in general, than is that of a comparable solution of a nitrate ester of an untreated starch. There is a decrease in viscosity with a decrease in the molecular weight of the parent molecule and an increase in viscosity with an increase in the grain size of the starch granule. Pretreatment may favour both of these changes.

The boiling of starch, under pressure, decreases viscosity to a minimum. Subsequent nitration of this pretreated starch, however, causes an increase in viscosity (cf. Table XXIX).[8]

The viscosity of the product obtained by treating starch with mixed acid reaches a maximum at the end of 1 hour, whereas at the end of 12 hours the viscosity has decreased to about one-half of the maximum. When nitric-phosphoric acid mixture is used as the nitrating agent for starch, the maximum viscosity of the product is not attained until at the end of about 12 hours. The viscosity of the esters obtained by the latter procedure is about ten times as great as is that of comparable esters prepared by the use of mixed acid.

The relative viscosities of 'amylopectin nitrate' and 'amylose nitrate' are shown in Table XXX.

TABLE XXX

Analysis and Viscosities of Comparable Nitrates of Starch Fractions

Type of nitrate	*Nitrogen*	*Viscosity at 20° C.*
	per cent.	poises
Amylopectin . .	11·82–12·05	28,000–31,000
Amylose . . .	13·40–13·45	61–84

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nitr e nitric acid esters of starch, in general, are soluble in acetic
ich a etone, chloroform, and mixtures of alcohol and ether, but
of ction uble in alcohol or in ether.
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hosphoric Acid Esters of Starch.—The phosphoric starch occur in nature.[44] The 'amylopectin' of example, contains about 0·07 to 0·09 per cent. ch is probably present in the form of an ester one phosphoric acid residue for every 212 to its.[44] When this 'amylopectin' is hydro-

lysed and the products separated by electrodialysis, considerable amounts of glucose-6-phosphate are obtained.[40] This suggests that the ester is principally of the 6- or primary type. The separation of this 'amylopectin' by electrodialysis is attributed to the strong anodic migration of the molecule as a consequence of the phosphoric acid residue.[17]

The first reported synthetic phosphoric acid ester of starch was prepared in 1919 by the treatment of a chloroform suspension of starch with phosphorus oxychloride in the presence of calcium carbonate.[27] The product was isolated as a calcium and as a lead salt of a phosphoric acid ester of starch or a degraded starch.[27] In 1927 phosphorus-free amylose was treated with phosphorus oxychloride in pyridine to give the corresponding ester.[45]

The phosphoric acid ester linkage in starch is hydrolysed very slowly by acid,[3] more readily by prolonged heating with or without acid,[47] and still more readily by alkali[46] or the use of amylophosphorylases.

IV. Sulphuric Acid Derivatives of Starch.—The first sulphuric acid derivative of starch was prepared in 1843 and isolated in the form of two lead salts, which were considered to be lead sulphonates of starch.[11] Two years later the 'calcium salt of starch bisulphate'[26] and other salts of the sulphuric acid esters of starch were prepared and analysed.[22]

The sulphuric-oleic acid derivative of both starch and dextrin was prepared in 1883.[32] This product was characterised as being water soluble, as forming metallic salts, and as undergoing hydrolysis to yield oleic acid, starch, and sulphuric acid.[32] In 1885 starch was treated with concentrated sulphuric acid and the product isolated as its barium, calcium, and lead salts.[30] The work was continued the following year, at which time consideration was given to the structure of the starch molecule.[30]

The sulphonation of vacuum-dried starch was effected in 1923 by the slow addition of the dry starch to a mixture of chlor[illegible] sulphonic acid, chloroform, and pyridine.[51] The derivative [illegible] isolated as its potassium salt, which was thought to be the salt [illegible] amylodisulphuric acid.[51] This derivative neither gave col[illegible] with iodine nor showed reducing action.[51]

A patent issued in 1928 covers the treatment of s[illegible] gaseous sulphur trioxide or fuming sulphuric acid [illegible] ducts, which are useful as protective colloids and a[illegible] in the synthesis of acetates, nitrates, or oth[illegible] During the following year, alkali starch was tre[illegible] sulphonic acid to give a derivative, which [illegible] mono ester.[24]

[illegible]it., [illegible]ROP, [illegible]32, 130. (See also No. [illegible] 1886, 7, 455. [illegible] 541. [illegible] *Helv. Chem. Acta.*, 1940,

The treatment of starch with concentrated sulphuric acid effects both degradation and esterification.[16] The former reaction is decreased and the latter increased by the use of an inert diluent such as chloroform, a relatively inert base such as pyridine, chlorosulphonic acid in place of concentrated sulphuric acid, and a careful control of the temperature.

V. The Xanthates of Starch.—The first reported xanthate of starch was prepared in 1907 by the slow addition of a 20 per cent. solution of sodium hydroxide, with vigorous stirring, to a suspension of dry starch in a mixture of carbon disulphide and toluene.[19] The product was dissolved in cold water and the resulting solution treated (*a*) with a dehydrating agent such as alcohol to precipitate the xanthate, or (*b*) with acetic acid and then with a solution of iodine to precipitate a dixanthate, in accordance with the equation :[19]

$$2\ \text{Starch}\cdot\text{O}\cdot\text{CS}\cdot\text{S}\cdot\text{Na}+\text{I}_2 \rightarrow \text{Starch}\cdot\text{O}\cdot\text{CS}\cdot\text{S}\cdot\text{S}\cdot\text{SC}\cdot\text{O}\cdot\text{Starch}.$$

The aqueous solution of the xanthates of starch, like that of the xanthates of cellulose, is yellow because of by-products such as sodium trisulpho-carbonate, which are decomposed readily by the addition of acetic acid before the treatment with iodine.[19]

Four years later (1911) starch was treated with a 5 to 20 per cent. solution of sodium hydroxide and then with carbon disulphide.[37] The mixture was allowed to stand at room temperature for 24 hours and then poured into alcohol to precipitate a sodium starch xanthate, which contained two equivalents of sodium and two equivalents of sulphur per glucose units.[37] A higher degree of xanthation is less readily attainable.

A patent issued in 1922 discloses the production of the xanthates of starch for use as adhesives in the fabrication of wood veneers.[50] The adhesive is produced by dissolving 15 kg. of sodium hydroxide in 75 kg. of water, and then adding this solution, slowly with t[illegible]ring, at 60° C. to a fine suspension of 200 kg. of starch in an as[illegible]al volume of water.[50] To this mixture is added, slowly with nitr[illegible]g and cooling, 7·5 kg. of carbon disulphide.[50]

[illegible]ich a[illegible]dy of the preparation and properties of the xanthates of of [illegible]action [illegible]s published in 1925,[55] a patent issued in 1932 covers mom[illegible] colo[illegible]on with diazonium salts to give an organic dithio-reactio[illegible]oiling [illegible]nd a patent issued in 1933 covers their use as textile at 70° [illegible] 14 pe[illegible]s.

disorgani[illegible] [illegible] of starch undergo the same general types of re-obtained i[illegible] extracti[illegible] corresponding xanthates of cellulose.[37] They [illegible]arch, 525 [illegible] fracti[illegible] degradation with an accompanying increase [illegible] their [illegible] accompanied by a decrease in the amount

of bound carbon disulphide. The xanthates of starch are decomposed, with the regeneration of carbon disulphide and starch, by treatment with glacial acetic acid, heat, or mineral acid.

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Chapter 16

OXIDISED DERIVATIVES OF STARCH

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The oxidation of starch has been considered from two viewpoints: (1) the effect of various oxidising agents under a variety of experimental conditions on the intact molecule as well as the mechanism of the oxidation, and (2) slight modifications, at a minimum cost, of the intact molecule in order to modify the physical properties and thus permit of new uses for these products. The first may be termed theoretical advances (I) and the latter industrial advances (II). The bulk of work of the former type, particularly until recently, has been done in educational institutions, whereas the most of the work of the latter type has been done in industrial laboratories and is available only in part in the scientific literature.

I. Theoretical Advances.—During 1875 the oxidation of starch by reagents such as bromine (followed by silver oxide, chromic acid, potassium permanganate, and nitric acid) was studied by Lieben and Reichardt.[61] A patent in 1892 claims that when starch is dissolved in an electrolyte such as a solution of hydrochloric acid, magnesium chloride, or sodium chloride and the mixture subjected to the passage of an electric current, a less viscous solution or complete solubility of the starch results.[45] One year later (1893) the chemical and physical properties of six starches were reported.[82]

The effect of bleaching powder, chloric acid, chromic acid, manganese dioxide, and potassium permanganate on starch was studied in 1896.[83] It was observed that starch treated with potassium permanganate forms a paste which is more liquid and transparent than is that prepared from ordinary starch.[83] Chlorates, in conjunction with an acid in the presence of a catalyst, such as copper sulphate or vanadium chloride, were observed in 1901 to effect the solubility of starch.[3] In the following year (1902) persulphates were used to solubilise starch.[12]

During 1905 Wolff prepared a modified starch (which is immediately soluble in dilute alkaline solution at 70° C.) by the treatment

of potato starch with acid oxidising solutions at room temperature.[96] Four years later (1909) the use of perborates to solubilise starch was reported.[28] A paper in 1921 indicates that starch is not attacked by chlorine dioxide (ClO_2).[84] It was observed in 1924 that Activin (sodium *p*-toluenesulphochloramide) effects the solubilisation of starch without appreciable degradation to dextrins or sugars.[27] Chloramines and oxidising agents were used in 1927 on cold swelling starches.[43] The story of starch, up to about 1928, is covered very thoroughly by Walton.[94] The oxidation of starch in the presence of ammonia and other bases yields a modified starch which is suitable for textile finishes.[65] The oxidation of the hydrolytic products, glucose and maltose, has been studied under a variety of experimental conditions but does not form a part of this restricted topic.[17]

Reagents used in the oxidation of starch include :[77] air,[73] bleaching powder,[21] bromine,[20] chloramines,[43] chloric acid,[83] chlorates,[3] chlorine,[35] chlorine dioxide,[84] chromic acid,[41] ferric chloride,[18] fluorine,[68] halogen,[1] hydrogen peroxide,[2] hypochlorite,[14] manganese dioxide,[83] nitric acid,[75] nitrogen dioxide,[64] perborates,[28] periodic acid,[11] persulphates,[12] silver oxide,[37] potassium permanganate,[58] *p*-toluene-sulphochloramide, and zinc oxide.[74]

(*i*) *Air as an Oxidising Agent.*—Air was used, in the presence of metallic ions in alkaline solution, in 1925, to effect the oxidation of starch.[73] The use of ferrous ion in alkali, for example, catalyses the oxidation of starch by air to yield carbon dioxide and water as the principal products.[73] Since the solution is alkaline, it is assumed that oxidation precedes any appreciable amount of hydrolysis of the starch molecule. Zinc oxide, in the presence of sunlight, also promotes the oxidation of starch.[74] Inasmuch as ozone has no apparent action on starch, it may be used to bleach starch or flour.[57]

(*ii*) *Chromic Acid as an Oxidising Agent.*—In 1893 it was observed that the oxidation of starch with chromic acid yields furfural (10 per cent.).[15] Such a drastic treatment is accompanied by much, if not complete, hydrolysis of the starch molecule. The action of chromic acid on starch was studied again in 1896.[56]

Haerz observed (1905) that an amylodextrin, which he prepared by the oxidation of starch with chromic acid, consisted of a number of molecular groups of different complexity and density of internal structure.[38] An erythrodextrin showed similar complexity, whereas an achrodextrin seemed to possess homogeneity.[38] Chromic acid was used (1905) to solubilise starch.[41]

In 1923 Reychler prepared a stable, soluble starch by the digestion of starch with a dilute solution of hydrochloric acid and

potassium dichromate, subsequent reduction of the dichromate with sulphur dioxide, filtration of the residue, washing, and drying.[78] When starch is treated with 0·25 *N* hydrochloric acid which contains 0·75 per cent. of potassium dichromate (or an equivalent weight of potassium permanganate) for 20 hours, washed, and dissolved in very dilute alkali, it gives a clear solution whereas water alone effects only swelling of the product.[79]

(*iii*) *Halogen as an Oxidising Agent.*—The first recorded observation relative to the oxidation of starch is by Liebig in 1829 that starch is slightly affected by the prolonged action of chlorine or chlorous acid.[60] In 1874 Habermann reported the isolation of gluconic acid (and dextronic acid) from the products obtained by the oxidation of starch with reagents such as bromine or silver oxide.[37] Seventeen years later (1891) Moissan reported that fluorine attacks starch with the deposition of carbon.[68]

Four years later it was noted that a starch suspension, after an hour's treatment with bromine in sodium hydroxide, reduces Fehling solution and gives a slight precipitate with phenylhydrazine, whereas neither bromine nor alkali alone produces such an effect.[13] Chlorine gas was used in 1902,[56] and a solution of chlorine and an alkali borate in 1926, to solubilise starch.[35] The use of various halogen compounds to solubilise starch is disclosed in the patent literature.[1] A 0·43 per cent. solution of hypochlorous acid effects an 80 to 90 per cent. solution of potato starch.[14]

(*a*) *Oxidation of Rx·CHO to Rx·CO·OH, by Halogen.*—It is known that, in general, aldehydes are oxidised more readily than are alcohols, and primary alcohols are oxidised (as a consequence of two available hydrogen atoms) more readily than are secondary alcohols. In the oxidation of the monosaccharides, there is considerable evidence indicative of the belief that the lactol ring remains intact in the normal oxidation of an aldohexose to the corresponding aldonic acid.[49] This suggests that the groups most susceptible to oxidation in the starch molecule are the terminal or free aldehyde groups.

It is generally believed, accordingly, that the acid groups produced during the mild oxidation of starch result from the oxidation of the terminal aldehyde groups to the corresponding carboxyl groups.[81] The oxidation of the aldohexoses, by use of alkaline hypohalites, to the corresponding aldonic acids is the basis of an analytical procedure.[95]

Alkaline hypochlorites are used in the commercial production of certain modified starches.[86] The nature and extent of oxidation is not known. It seems relatively certain that some of the initial

oxidation products are not uronic acids.[23] Hypoiodite in methanol, for example, is used to oxidise the aldomonosaccharides to the corresponding aldonic acid for subsequent characterisation as derivatives of benzimidazole.[69] This reaction has been used also to determine the relative chain length of dextrins, which are produced by the action of α-amylase[70] and of hydrochloric acid on corn starch.[59]

(*b*) *Oxidation of* $Rx \cdot CH_2 \cdot OH$ *to* $Rx \cdot CO \cdot OH$, *by Halogen.*—An *amylodextrinic acid* was obtained during 1925 by the oxidation of *amylodextrin* with bromine in the presence of a mild base such as barium carbonate.[89] The product gave a positive naphthoresorcinol[92] test and yielded furfural when distilled with hydrochloric acid.[89] This derivative is considered to be a polyanhydroglucuronic acid.

When bromine is used as the oxidising agent, as much as half of the oxidised starch may be polyanhydroglucuronic acids.[23] The cinchonine salt of glucuronic acid, obtained by the acid hydrolysis of an oxidised starch, has been isolated and characterised.[22] These products appear to be somewhat comparable to those obtained by the oxidation of starch in an organic solvent by the use of nitrogen dioxide.[64]

(*c*) *Oxidation of* $Rx \cdot CHOH \cdot Rz$ *to* $Rx \cdot CO \cdot Rz$, *by Halogen.*—In certain oxidation reactions of the aldohexoses, it is believed that α-ketoaldonic acids are formed as intermediates.[48] Such intermediates are probably formed in the air-oxidation of the simple sugars in the presence of an alkaline solution of iron pyrophosphate.[16] It is to be expected, then, that under certain oxidative conditions, some of the secondary hydroxyl groups in the starch molecule will be oxidised to ketone groups.

Bromine in acid solution has been studied as an oxidising agent on about 50 carbohydrates.[20] In the case of starch, the data indicate some oxidation of the secondary hydroxyl groups to ketone groups.[20] This type of oxidation is indicated also by the formation of oximes by certain oxidised starches[22] and by the reducing power of some oxidised starches, at certain stages of the oxidation, against Fehling solution.[23] The nitrogen analysis on the oxime of a certain oxidised starch is equivalent to about three ketone groups for every four anhydroglucose units.[22]

(*d*) *Oxidation of* α, β-*Secondary Alcohol Groups to Carbonyl or Carboxyl Groups by Halogen.*—Under proper experimental conditions, particularly in the use of periodic acid, the α, β-hydroxyl groups in a polyanhydroglucopyranose structure are oxidised to ketone groups with subsequent cleavage and oxidation to yield carboxyl groups. A decrease in optical rotation and an

increase in the calcium content of the acid salts obtained, when starch is oxidised with bromine, indicate this particular type of oxidation and cleavage. Upon further oxidation and hydrolysis of these products, salts of diacids are obtained, which contain less than six carbon atoms.[22] This general type reaction is believed to take place also in the production of the hexachlorostarches,[6] which have been considered in a previous chapter (Chapter 15, p. 333).[6]

(*iv*) *Nitric Acid and Nitrogen Dioxide as Oxidising Agents.*—In 1838 Pelouze showed that oxalic acid is one of the products obtained by the oxidation of starch with concentrated nitric acid.[75] During 1892 Petit subjected starch to prolonged treatment with nitric acid at 40° C.[76] Addition of water to the mixture yielded carbon dioxide, oxides of nitrogen, and a reducing acid ($C_5H_6O_5$).[76] This acid, on prolonged boiling, yielded a new acid ($C_5H_8O_6$).[76] These products suggest that hydrolysis precedes or accompanies oxidation. The reaction may be, essentially, one for the oxidation of monosaccharides.

Mench and Degering, in 1942, oxidised dry starch, suspended in an inert organic solvent, with nitrogen dioxide to the polyglucuronic acid.[64] By this laboratory procedure 16·5 gm. (0·1 mole) of dry starch is added to 150 ml. of carbon tetrachloride in a 250 ml. three-neck flask, which is fitted with an efficient mechanical stirrer, a reflux condenser, and a water-jacketed burette.[64] The nitrogen dioxide, in calculated amount from density and temperature data, is added dropwise through the burette, accompanied by efficient agitation of the mixture.[64] The temperature is maintained at 25 to 29° C. until the oxidation is complete. The product is then filtered on a pyrex fritted-glass funnel, washed well with carbon tetrachloride, freed of solvent on a vacuum filter, washed well with distilled water until free of traces of acid, washed with acetone, and then washed with ether. The product is then dried in a dessicator over phosphorus pentoxide. (Yield, 16 or more gm.)

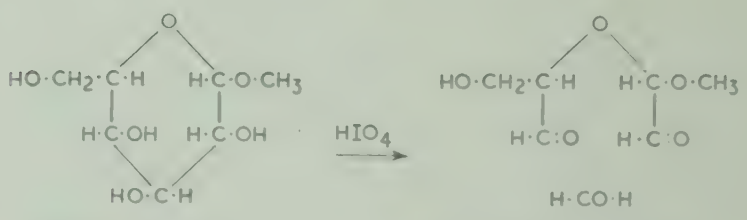
The carboxyl content is determined by titration with standard alkali,[93] and the polyglucuronic acid is identified qualitatively by use of the β-naphthol test.[92]

These oxidised starches, even those with relatively low carboxyl content, are insoluble in water but dissolve readily in warm, dilute bases such as dilute ammonium hydroxide, aqueous pyridine, aqueous Triton B, aqueous sodium carbonate (2 per cent.) aqueous sodium hydroxide (2 per cent.), and similar bases. They dissolve in a 5 per cent. solution of sodium bicarbonate with the liberation of carbon dioxide. They show no sign of retrograda-

tion when relatively concentrated solutions of their salts are cooled. When these solutions are poured into alcohol, precipitation of the salts results. Salts of the heavy metals are obtained by treatment of the oxidised starches with aqueous solutions of the metal acetates.

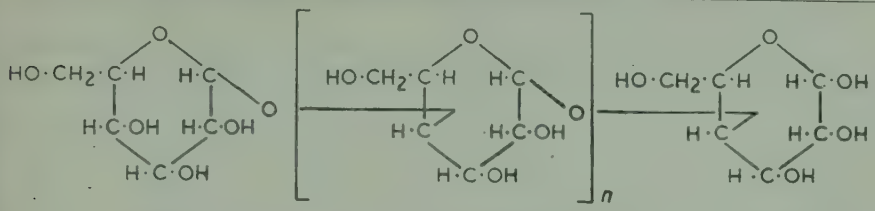
The salts of these polyanhydroglucuronic acids may replace certain other modified starches as dispersants, gel-formers, stabilisers, thickening agents, and in similar uses. The free acids may be used as acidulants, as distintegrates in certain food and medicinal products, as dispersants, and as a source of glucuronic acid.

(*v*) *Periodic Acid as an Oxidising Agent.*—The first use of periodic acid as an oxidising agent in carbohydrate chemistry was by Malaprade in 1928 in a study of the ring structures of various methyl glycosides.[63] These studies led to the conclusion that the alkyl aldohexopyranoside molecule is oxidised to a dialdehyde with the formation of one mole of formaldehyde per aldose unit, in accordance with the equation :[44]

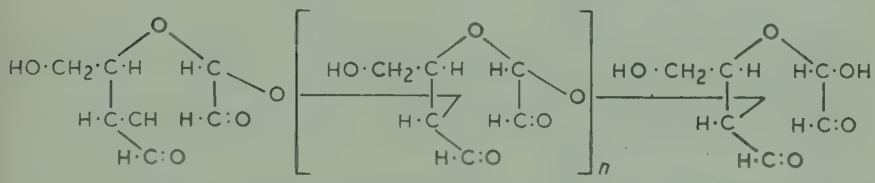


This postulate was confirmed in 1936 by Jackson and Hudson, when they oxidised methyl *α-D*-mannoside with aqueous barium hypobromite to a diacid, which was isolated and characterised as its strontium salt ($C_6H_8O_7Sr$).[50] Three other methyl *D*-glucosides gave the same derivative.[50] This salt is obtained also by the oxidation of the glucoside with periodic acid to the 2, 4-dialdehyde and subsequent oxidation with bromine in aqueous strontium carbonate.[50]

The treatment of ungelatinised corn starch with an aqueous solution of hydriodic acid (0·58 M) at room temperature for 24 hours, results in the reduction of about one molecular equivalent of hydriodic acid per glucose unit.[51] This is the theoretical amount required to effect oxidation of the starch to the dialdehyde stage. Continued treatment with the oxidant results in a greatly diminished rate of oxidation, which indicates that the initial stage of the oxidation is complete at the dialdehyde stage.[51] The equation for the reaction may be formulated as :



Starch, of the amylose or continuous chain type



The corresponding dialdehyde of the continuous chain type

This oxidised starch is very similar, when viewed under a microscope, to the unoxidised starch except that the oxidised product shows no birefringence.[51] The dialdehyde derivative is insoluble in cold water but dissolves in hot water. It forms an amorphous precipitate with phenylhydrazine at 25° C., has a specific rotation in sodium light of + 9 at 20° C., is not hydrolysed by malt diastase, and is negative to the Wagner reagent (I_2/KI, aq.) but positive to Fehling solution. A transparent film is produced by the evaporation of a thin layer of an aqueous solution of the product.[51]

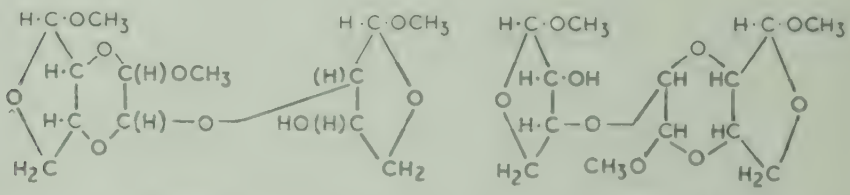
The hydrolysis of the dialdehyde starch with hot, dilute hydrochloric acid (0·1 N), gives a solution with optical activity, which closely approximates that of a comparable solution of L^+-erythrose (−13 vs + 4).[51] It is postulated, accordingly, that the optical activity of the dialdehyde starch may be attributed to the L^+-erythrose residue.[51]

Subsequent studies have indicated that the terminal glucose unit is further oxidised to give 2 moles of formic acid and 1 mole of formaldehyde whereas the terminal glucosyl unit yields 1 mole of formaldehyde.[11] Subsequent hydrolysis, then, should yield 2 parts of formaldehyde, 2 parts of formic acid, 1 part of glyceraldehyde, 1 part of malonaldehyde, $n + 1$ parts of glyoxal, and n parts of L^+-erythrose.

The determination of the formaldehyde formed in this reaction has been used in estimating the number of glucose units per dextrin or starch molecule.[10] These values agree very well with those obtained through an evaluation of the reducing properties of certain dextrins and starches.[80] These data suggest, moreover, that the fundamental starch unit contains somewhat more than the 25 glucose units previously proposed from a consideration of methylation studies of starch.[42]

Both the glyoxal and the erythrose have been isolated, in the form of derivatives, from the periodic acid oxidation of starch, which is additional confirmation for the proposed course of this oxidation.[10] Comparable results are obtained with raw granules and gelatinised starch, both with respect to reaction time and the nature of the final product.[31] It is apparent, therefore, that the periodate ion can penetrate into the intact granule and attack the susceptible groupings.

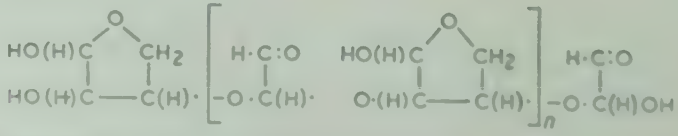
The methanolysis of periodate-oxidised starches yield about 2 per cent. of a crystalline, leavoratory, white derivative (M.p. 148° C.) of unknown composition.[4] This product, which is positive to Fehling solution after mild hydrolysis, is obtained from the periodate-oxidised arrowroot, corn, potato, soluble potato, and wheat starch. It is thought to be the methyl acetal of an aldehyde or ketone.[4] It has been assigned an empirical formula of $C_{10}H_{12}O_5(OH)(OCH_3)_3$ and one or both of the following graphic formulas;[66]



Formula A Formula B

These proposed formulas consist of a methylerythrofuranoside unit, which is combined in a 1, 4-dioxane ring with a glyoxal residue, which is linked in turn to another methylerythrofuranoside residue. Continued methanolysis, with subsequent steam distillation, yields some of the tetramethylacetal of glyoxal, which is consistent with the proposed formulation. The formation of a monoester with *p*-toluenesulphonyl chloride, together with the resistance to hydrolysis shown by this ester, indicate that there is one available hydroxyl group, which is probably of the secondary type.[72]

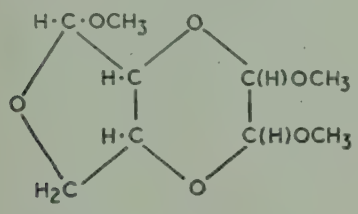
In the formation of these ring structures it is assumed that the erythrose units of the oxidised starch follow the normal tendency of erythrose to cyclise to a furanoid ring. If this tendency is general, the rearranged structure of the periodate-oxidised starch may be represented as:[47]



Proposed structure of rearranged periodate oxidised starch

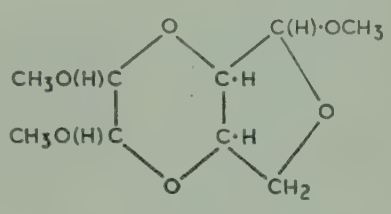
The methanolysis of a derivative of this type can give rise to products of the type proposed in Formula A and B, each in eight isomeric forms.[53] To date only about 22 per cent. of the oxidised starch can be accounted for by derivatives of this type.[53]

Michell and Purves have isolated another white, crystalline derivative (M.p. 97-98° C. $(a)_D^{20} = -59 \cdot 1°$, M.W. in CCl_4, 212), which they formulate as:[67]



Formula C

and/or



Formula D

Products A and B, moreover, have been degraded to products C and D, which is further indication of the probability of the correctness of these assigned structures.

The failure to isolate any appreciable amount of erythrose from the acid hydrolysate of these mixtures, may be attributed to subsequent condensation with glyoxal to yield unidentified products.[46] Analogous condensations are known to occur. About 90 per cent. of the glyoxal units theoretically present in the periodate-oxidised starch, according to these postulates, can be recovered.[33]

(*vi*) *Permanganate as an Oxidising Agent.*—In 1868 Langbein showed that 2 gm. of starch will effect the reduction of 3 gm. of potassium permanganate, but made no attempt to identify the end products of the reaction.[58] The treatment (1890) of starch with potassium permanganate, in varying concentrations, results in the evolution of carbon dioxide, the precipitation of manganese dioxide, and the formation of a series of gummy acids, which may be precipitated by barium hydroxide or by lead acetate.[62]

A soluble starch is prepared by the treatment of starch with a solution of potassium permanganate, then with dilute hydrochloric acid, and finally with sulphur dioxide or dilute bisulphite solution to remove the excess chlorine.[87]

Urea was obtained (1921) in a 20 per cent. yield by heating starch in an aqueous solution of ammonical copper oxide and calcium permanganate.[25] The oxidation of starch (1922) with calcium permanganate, in the presence of ammonium hydroxide and mercuric oxide or silver nitrate, yields hydrogen cyanide.[26] A clear, semi-fluid, transparent paste is obtained by the treatment of starch with dilute permanganate solution and subsequent decoloration with sulphur dioxide.[88]

(*vii*) *Peroxide as an Oxidising Agent.*—Wurster[97] observed, in 1889, that starch is not affected by hydrogen peroxide at room temperature but that it is hydrolysed into glucose and dextrin by hydrogen peroxide in boiling acid or alkaline solution.

Asboth[2] reported in 1892 that the treatment of starch with hydrogen peroxide, in the presence of ammonia, results in liquefaction with the evolution of carbon dioxide and oxygen. The reaction mixture is filtered and the products in the filtrate precipitated fractionally by the use of alcohol and barium hydroxide.[2] Five years later (1897) soluble starch was prepared by the treatment of starch with a cold solution of sodium peroxide.[90] An increase in rotation was observed with an increase in the concentration of the sodium peroxide.[90]

In 1898 the solution of starch was effected by the use of sodium peroxide,[91] and 11 years later (1909) the use of acids, alkalis, and salts were suggested as catalysts in the oxidation of starch with hydrogen peroxide.[24] It was observed that hydrogen peroxide alone, in large excess, effects complete liquefaction. During 1910, achrodextrin, erythrodextrin, and maltose were obtained by the action of a dilute solution of hydrogen peroxide on starch at 37° C.[30]

The action of hydrogen peroxide on starch affects the activity of amylase.[31] Strong, transparent sizes, which are insoluble in water after drying, are obtained by the proper treatment of starch with neutral hydrogen peroxide.[32] In 1913 starch was solubilised by hydrogen peroxide in the presence of ferric chloride, but when these reagents are used individually no appreciable change occurs.[19]

A mixture of ferric chloride and hydrogen peroxide, was used in 1913, to effect an appreciable degradation of starch, whereas the affect of either used singly is much less.[18] Six years later (1919) a paper reports that 60 per cent. aqueous hydrogen peroxide solution dissolves starch to give dough-like products or viscous solutions.[5] In 1936 the hydrogen peroxide oxidation of starch, in the presence of ferrous ion, was studied by Brown, who concluded that hydrolysis precedes oxidation and that the reaction does not represent a true oxidation of the starch molecule.[8]

II. Industrial Advances.—The commercially oxidised starches, in general, show the same (1) colour reactions with iodine, (2) granule structure, (3) insolubility in cold water, and (4) polarisation crosses as does the raw starch. When heated with water, however, the former give solutions of varying degrees of fluidity whereas the latter give pastes or gels.

The extent of modification of an oxidised starch is usually

expressed in terms of fluidity. Fluidity is defined as the volume of liquid in ml. that will pass through a given orifice under a specified set of conditions in the same time interval required for 100 ml. of water (see Chapter 19, Vol. II).

The oxidised starches have (1) greater adhesive strength, (2) higher fluidity values, (3) lower rates of congealing, (4) shorter cooking times, and (5) suspensions of lower turbidity than do the parent starches. Films obtained from the former are tough and horny in contrast to those of the latter which are very brittle.

The most important uses for the oxidised starches are in the sizing of cloth, paper, and raw cotton yarns. Cloth is sized principally for appearance and ' feel '. Paper is treated with starches and highly oxidised starch gums to close up the pores, to increase the bursting, fold, and tensile strength, to give ' feel ' and rattle, and to lay the fuzz and produce a smooth surface (see Chapter 11, Vol. II).

Cotton yarns, which contain no size, have neither the strength nor the resistance to abrasion to withstand looming when used as warp. If the warp threads are treated, however, with a proper mixture of gums, lubricants, softeners, and starch, they acquire the necessary elasticity, pliability, stiffness, and strength to withstand the wear and strain of weaving. The oxidised starches are the most satisfactory for this treatment with respect to both the ease of handling and the properties of the sized warp threads (see Chapter 12, Vol. II).

The changes resulting from the oxidation of starch for commercial uses may be defined in terms of (1) birefringence, (2) digestibility by β-amylase, (3) gel strength, (4) reducing power, (5) rigidity, (6) status of swollen granules, (7) viscosity, and (8) volume of swollen granules. With an increase in the amount of oxidation by hypochlorite solution, there is a decrease in gel strength, rigidity, swelling, and turbidity, but an increase in the reducing power of the product and a marked change in the appearance of the granules under the microscope.[22] The birefringence of the starch granules do not change, by oxidation, when up to as much as one-half equivalent of chlorine per glucose unit is used.

The agents most commonly used in the commercial oxidisation of starch are the alkali hypohalites, particularly calcium and sodium hypochlorite. What is commonly known as a chlorinated starch is one which has been oxidised by the use of chlorine in the presence of alkali. Under these experimental conditions the essential reaction is one of oxidation rather than one of substitution.

The usual practice is to treat an aqueous suspension of starch

(36 per cent.) with hypochlorite solution (6 to 8 per cent. active chlorine), which contains a slight excess of alkali. Since the over-all reaction is exothermic, the hypochlorite solution is added slowly while the reaction temperature of the mixture is maintained at about 90 to 125° F. The progress of the reaction is followed by withdrawing samples from time to time for determination of fluidity. When the product has the desired fluidity, the reaction is stopped by the addition of sodium bisulphite or other antichlor agent, the slurry is adjusted to a *p*H of 3 to 7, and the product filtered, washed, and dried. The properties of the finished product are a function of the amount of hypochlorite used, and the alkalinity, temperature, and time required for the reaction.

A modification of the process consists of the addition of a small amount of hypochlorite to the starch slurry just before the final removal of water, in order to oxidise the sulphur dioxide from the previous treatment.[9] This procedure yields a starch of higher viscosity.[9] Refined sago starch products are produced by the oxidation of crude sago flour with hypochlorite.[29]

The treatment of starch with a 10 per cent. solution of sodium hypochlorite, or other highly concentrated oxidising liquid, causes a sufficient increase in the temperature of the mixture to evaporate the moisture and leave a nearly dry soluble starch as the residue.[36]

If the oxidation is effected by the use of halogen in slightly acid solution, with proper conditions with respect to temperature, time, and amount of halogen, the product has a low cold paste viscosity and a maximum hot paste viscosity.[54] The treatment of a starch slurry (30 per cent.) with bromine (or chlorine) at 75° F., yields a thick boiling, thick setting product, which can absorb more water than the raw starch.[54] Subsequent acid hydrolysis gives a paste that is less congealing than a comparable paste made by acid hydrolysis alone.[54]

The grinding of air-dry starch with 3 per cent. of its weight of calcium hypochlorite is claimed as a solubilising procedure for starch.[21] By another dry process, dry starch is placed in a water-jacketed mixer and sprayed, accompanied by thorough mixing and cooling, with a solution of hypochlorite (8 per cent. available chlorine, 2 per cent. sodium hydroxide) at a temperature of about 113° F.[71] The product is then dried to a moisture content of 10 to 20 per cent.[71]

By another dry oxidation technique, dry starch is treated with chlorine gas.[7] The claims cite that ' by varying the temperature of treatment, the length of the treatment, which may vary from 1 to 12 hours, and the amount of chlorine used, it is possible to produce any modified starch product ranging from thin boiling

starches, substantially insoluble in cold water (1 per cent. soluble), to dextrins which have solubilities in cold water of over 90 per cent.'[7] It is likely that this process involves both oxidation and substitution.[6]

Oxidised starches may be produced by the addition of ammonium persulphate to a starch slurry and then treatment with acetic or hydrochloric acid at room temperature.[52] When the desired degree of oxidation is attained, the product is filtered, washed, and dried. The ammonium persulphate may be replaced by sodium perborate. In this dry process, about 1 per cent. of the perborate or the persulphate is mixed with the dry starch, the mixture suspended in water, and then cooked to a paste.[52] Only moderately modified starches are usually obtained by these treatments.

Barium peroxide produces little or no modification when (1) it is mixed with dry starch or (2) added to an aqueous suspension of starch at room temperature.[40] Heating of the aqueous suspension, however, produces marked modifications of the starch.[40] A thin boiling but thick setting starch may be prepared by the treatment of a starch slurry with calcium peroxide at 126° F. for about 24 hours.[55] The oxidation mixture (*p*H of about 11) is then neutralised, filtered, suspended in water, filtered, washed, and dried.[55] A starch free from thermophiles, with little or no modification otherwise, is obtained when moist starch is treated with 0·05 to 0·15 per cent. hydrogen peroxide at 100 to 110° F. and then dried in a Buell drier.[85]

A decrease in viscosity and an increase in transparency of starch pastes may be obtained by the treatment of the starch, before pasting, with alkaline permanganate. The modification may be continued until products are obtained which give a red stain with iodine. The yellowish tinge of the oxidised starch, which is due to traces of manganese compounds, may be bleached by treatment with agents such as oxalic acid, sodium hydrosulphite, or sulphur dioxide followed by thorough washing. Sodium *p*-toluenesulphonchloramide, chloramine-T, also known as Aktivin, is widely used in Europe to effect the solubilisation of starch without the attendant formation of dextrins or gums.

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Oxidation by Chromic Acid or Permanganates in Acid Media

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J. M. EDER, *J. prakt. Chem.*, 1879, **19**, 294. (Obtained dextrins.)
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V. I. NAZAROV, *Colloid J.* (U.S.S.R.), 1940, **60**, 254. (Oxidation with permanganate takes place throughout granule and follows closely the equation for a unimolecular reaction.)

Oxidation with Hydrogen Peroxide in Acid Media

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Patents on Peroxide Oxidation in Acid Media

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C. GERBER, *Compt. rend. soc. biol.*, 1912, **72**, 1002; 1912, **73**, 356 and 358. (Pretreatment with 300-500 pts. of chlorine or bromine per million accelerated amylase hydrolysis.)
C. H. BAILEY, *Chemistry of Wheat Flour*, pp. 212, 220 *et seq.* Chemical Catalogue Co., N.Y., 1925. (Bleaching wheat flour with chlorine increases enzymic activity.)
FLETCHER and T. C. TAYLOR, *J. Amer. Chem. Soc.*, 1938, **60**, 3018. (Action of chlorine water on β-amylose.)
ANON. T.I.B.A., 1935, **13**, 633. (Halogen compounds used for oxidation.)

Patents on Oxidation of Starch by Chlorine

BERNARDI, Ital. P., 126,803, 1913; *C.A.*, 1915, **9**, 2160.
FULLER, U.S.P. 2,014,799, 1935.
HARTWIG, U.S.P. 798,509, 1905.
KINDSCHER, G.P. 149,588, 1904; *Chem. Zentr.*, 1904, I, 976. (Chlorine on moist starch to solubilise the latter.)
J. R. MACMILLAN, U.S.P. 1,567,609, 1925.
MORI, Japanese P. 100,061, 1933; *C.A.*, 1934, **28**, 2564.
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Oxidation with Iodine

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VINTILESCU and FALTES, *Bul. soc. chim. România*, 1923, **5**, 59; *C.A.* 1924, **18**, 1475. (Oxidation of dextrin by heating at 100° C. with iodine discussed.)

Oxidation with Oxyhalogen Acids

BRÄUTIGAM, *Pharm. Ztg.*, 1901, **46**, 636. (Hypochlorous acid on starch.)
G. B. JAMBUSERWALA and K. R. KANITKAR, *J. Text. Inst.*, 1939, **30**, 85T. (Measured change in chemical and physical values of corn, wheat, sago, and potato starches treated with acidified hypochlorite.)
W. SECK, E.P. 437,890, 1935; *C.A.* 1936, **30**, 3270. (Oxidation by hypochlorous acid.)
R. HAAKE and W. HAAKE, G.P. 164,385, 1905; E.P. 19,720, 1901; *Chem. Zentr.*, 1905, II, 1566. (Soluble starch by action of chloric acid.)
MIROW, G.P. 273,235, 1914; *C.A.* 1914, **8**, 2825. (Soluble starch by action of chloric acid.)
JACKSON and HUDSON, *J. Amer. Chem. Soc.*, 1937, **59**, 2049. (Periodic acid reacts quantitatively with starch.)
GRANGAARD, MICHELL and PURVES, *J. Amer. Chem. Soc.*, 1939, **61**, 1290 (Oxidised starch in methyl alcohol containing HCl.)

Oxidation by other Per-compounds

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R. HALLER, *Helv. Chim. Acta*, 1940, **23**, 596. [(Perborate and persulphate used for slight physical modification of starch.)
BROOKS, U.S.P. 779,583, 1905.

N. Cerf, U.S.P. 698,632, 1902.
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F. Fritsche, E.P. 1,351, 1908; *C.A.* 1909, **3**, 600.
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Holste, E.P. 30,390, 1909; *C.A.* 1911, **5**, 2444.
Kesseler and Döring, G.P. 535,839, 1931; *C.A.*, 1932, **26**, 1150.
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J. S. Reichert and S. A. McNeight, U.S. Applic. 343,455, 1/7/1940. (Starch treated with aqueous organic mono-peracids. Citations against by U.S. Patents Office: Debuigne, F.P. 777,722, 1935; Flick, E.P. 25,121, 1909; Stoddard, U.S.P. 1,718,609, 1,687,805 and 1,687,804; McKee, U.S.P. 1,767,543.)

Oxidation by Hypohalites in Alkaline Media

R. Haller, *Kolloid-Zeit.*, 1927, **41**, 81. (Temperature rise depends on quantity of sodium hypochlorite used.)
Trotman, *Dyer*, 1928, **60**, 160. (Hypochlorite or ammonium persulphate for preparing textile starches.)
Rassow, *Melliand Textilber.*, 1931, **12**, 468. (Measures temperature rise, decrease in viscosity and increase in reducing power.)
Lobenstein, *Thesis*, Leipzig; *C.A.*, 1931, **25**, 3867. (Soluble starch by alkaline sodium hypochlorite.)
J. R. Katz, *Textile Research*, 1939, **9**, 146. (Extensive list of patents on hypochlorite modification given.)
De Chalmot, *Amer. Chem. J.*, 1895, **17**, 535. (Soluble starch using bromine and sodium carbonate.)
Hönig and Ruziczka, *Biochem. Zeit.*, 1930, **218**, 397. (Bromine and barium hydroxide solution on starch gave maltobionic acid barium salt.)
Reisz, Hungarian P. 104,972, 1933; *C.A.*, 1933, **27**, 4123. (Soluble starch using bromine as oxidant.)
Dubeau, MacMahon and Vincent, *Amer. Dyestuff Reptr.*, 1939, **28**, 590. (Sodium chlorite used to remove starch in kier boiling.)
Kihara, *J. Agr. Chem. Soc.*, Japan, 1939, **15**, 107; *C.A.*, 1939, **33**, 6253. (Uses bromine and chalk to oxidise starch.)
Syniewski, *Rocnziki Chem.*, 1922, **2**, 83; *C.A.*, 1923, **17**, 1627. (Oxidised amylodextrin with bromine in presence of barium carbonate.)

Patents on Modification by Alkaline Hypochlorite

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Bryant, U.S.P. 1,937,543, 1933.
Deutsche Maizena G.m.b.H., F.P. 771,029, 1934; *C.A.*, 1935, **29**, 952.
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Hildebrandt, G.P. 545,081, 1932; *C.A.*, 1932, **26**, 3135.
F. Höppler, G.P. 558,145, 1932; *C.A.*, 1933, **27**, 1229.
Kesseler and Döring, G.P. 535,839, 1931; *C.A.*, 1932, **26**, 1150.
Montgomerie, E.P. 490,070, 1938.
National Adhesives Corp., F.P. 729,259, 1932; *C.A.*, 1932, **26**, 6176.
Reisz, Hungarian P. 104,972, 1933; *C.A.*, 1933, **27**, 4123.

SCHOTZ, E.P. 345,985, 1931 ; *C.A.*, 1931, **25**, 5477.
SPROCKHOFF, G.P. 618,265, 1935 ; *C.A.*, 1936, **30**, 319.
SUTHERLAND, E.P. 121,943, 1919 ; *C.A.*, 1919, **13**, 1109.
VAN DER MEULEN, G.P. 624,988, 1936 ; U.S.P. 2,053,012, 1936 ; *C.A.*, 1936, **30**, 7378.
VON BRUEDER, U.S.P. 646,724, 1900.
I. G. FARBENIND., G.P. 538,839, 1924. (Air dry starch ground with 3 per cent. its weight of calcium hypochlorite.)
CORN PRODUCTS REF. CO., E.P. 530,503, 16/11/1939. (Starch chlorinated in dry state with hypochlorite.)
M. D. ROZENBROEK, E.P. 534,112, 27/12/1938. (Powdered starch and alkaline reagent mixed and chlorine passed in.)

Action of Miscellaneous Oxidising Agents in Acid Solution

SAMEC and ULM, *Kolloidchem. Beih.*, 1936, **43**, 287. (Chlorine dioxide does not change appearance of starch granules but some chemical changes take place.)
MASSOL, *Compt. rend.*, 1911, **152**, 902. (Oxidised soluble starch by irradiation.)
BIELECKI and WURMSER, *Biochem. Zeit.*, 1912, **43**, 154. (Irradiation of soluble starch solutions gave splitting and oxidation.)
ONO, *Rev. Phys. Chem.*, Japan, 1940, **14**, 25 ; *C.A.*, 1940, **34**, 5721. (Some oxidation in starch pastes disintegrated with ultrasonic waves.)
BUCHSWEILER AKT.-GES., Administration der Minen, G.P. 227,606, 1910 ; *C.A.*, 1911, **5**, 2200. (Starch oxidised by air, in acid solution.)
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Chapter 17

REACTIONS WITH FORMALDEHYDE

The action of formaldehyde on starch has received much attention commercially, and work has been directed mainly towards producing substances for use as adhesives, textile agents or antiseptic agents, and it may be of interest to examine this work briefly.

According to Classen, when starch or dextrin is treated with formaldehyde, compounds of constant composition are formed[1] which are comparatively stable to heat, and which may, or may not, form gels, according to the conditions of preparation. Upon treating with dilute acid or alkali, free aldehyde is liberated. These white compounds have been suggested for use as antiseptic surgical dressings by the above worker[2–4] and others.[5, 6]

C. Longard[7] held that the compounds formed are mechanical mixtures of starch and formaldehyde, but further work tends to show that a chemical entity is concerned, very possibly by cross-linkage, via a methylene group, between two hydroxyl groups in different molecular chains.

Potato starch and amylodextrin, on treatment with 40 per cent. formaldehyde for two months,[8] give no reaction with iodine, and yield compounds that rapidly decompose on heating with liberation of formaldehyde. These compounds are hydrolysed slowly by water and rapidly by acids, the product of hydrolysis resembling amylodextrin. The temperature of gelatinisation is stated to decrease with increase in concentration of formaldehyde and the length of time of the action.[9]

Removal of the formaldehyde from the white compounds regenerates the starch practically unchanged.[10, 11] and addition of ammonium acetate to ' amyloform ' solution[12] causes the starch to give the normal blue colour with iodine, whilst if alcohol be added to the amyloform solution, practically unchanged starch is precipitated. Formaldehyde is also regenerated by treatment with phenylhydrazine and alcohol.[14] W. von Kaufman and A. Lewite[13] considered that the formaldehyde produces a reversible physical change in the state of aggregation of the starch, although G. Woker and H. Maggi[15–19] contend that the starch is hydrolysed by formaldehyde.

According to H. Sallinger,[20] amylodextrin, after digestion with formaldehyde, showed no change in its optical activity, and soluble starch converted to amyloform and then regenerated showed no increase of reducing power. To explain why he found hydrolysis and degradation to occur, whilst other workers obtained unaltered starch, G. Woker[16, 19] suggests that under the conditions used by the other workers resynthesis of the degradation products occurred, and he cites as an analogous phenomenon the digestion of egg albumin by papain.

M. Samec[21] has studied the reaction at various temperatures, and finds that a loose addition- and highly-hydrated compound is formed, giving no iodine reaction and having about the same molecular weight and dialysable fraction, considerably higher viscosity, slightly higher specific gravity, and slightly lower optical rotation than starch itself. He considers that formaldehyde is incapable either of degrading starch or of resynthesising its degradation products. The action of formaldehyde appears to be the same on both amylose and amylopectin.

The author has found that although the compound formed when 38 per cent. formaldehyde is added to starch in slight excess gels in the cold, it becomes insoluble in boiling water. Potato starch gives a clear gel, cassava starch a slightly cloudy gel some time later, whilst wheat and maize form opaque pastes on standing in the cold for several hours. If a large excess of formaldehyde be used, especially with starch previously gelled, a type of product soluble in cold water is obtained, a still different type being produced by the action of formaldehyde when present in less than the theoretical amount. Treatment of starch for several days in the cold with 1 per cent. hydrochloric acid and 5-10 per cent. formaldehyde gives products which do not gel even in boiling water. The granules from potato after boiling appear as swollen, but very wrinkled sacs.

If a soluble starch giving a limpid solution is used, the product no longer disperses and fluid solutions in hot water are not obtainable; instead, it swells up at the temperature of gelatinisation of the original untreated non-soluble starch. An interesting product is that obtained by the action of formaldehyde on dry non-soluble starch, which gives a thick viscous solution at 55° C. It may be prepared[24] by heating 100 pts. of starch with 1·8 pts. of formaldehyde for 1 hour at 76° C. in a closed vessel with stirring. By varying the conditions of reaction, the temperature of gelling and the nature of the solution can be considerably altered.

For example, if the above product is pasted with 1½ times its weight of water at 55° C., dried at 80° C. and powdered, a cold-

water compound is obtained which with 12-14 times its weight of water gives a highly adhesive transparent jelly.

E. Blumer[22] treats starch with diluted alkali hydroxide of 5° Be. and formaldehyde for several hours, and then heats for a short period; after washing the product free from formaldehyde and neutralising with dilute acetic acid, he dries it at a temperature not exceeding 50° C. Other patents[25] describe the production of a starch product resistant to water by the action of formaldehyde and an acid on dehydrated starch. The colour, opacity and adhesiveness of the products vary according to the conditions of the reaction and the type of starch employed. G. J. Leuck uses anhydrous starch to prepare formaldehyde starch for use as a plywood adhesive where the presence of the minimum amount of water is desirable.[26]

The action of formaldehyde on starch has been summarised by F. Beltzer.[23]

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PART II

AMYLASES AND THEIR ACTION ON STARCH

CHAPTER 18

GENERAL FEATURES AND NOMENCLATURE OF AMYLASES

Occurrence.—The enzymes capable of hydrolysing starch to simpler products are known generally as 'Amylases' and they occur very widespread in nature. Kirchhoff discovered 'diastase' in 1815 and his work was followed in 1833 by that of Payen and Persoz who named the active principal in barley malt 'diastase', followed its action on starch by the iodine-reaction and even effected some concentration and purification of the amylase with alcohol. The conversion of starch into sugar by saliva was noted in 1831,[1] although the significance of the reaction was not then fully grasped.

Since that time amylases have been found to occur in yeasts, the small intestine, in the duodenum,[266, 267] the blood,[268] liver and pancreas of the human body[2] and of animals, in the muscle,[107–110, 240] in dogs' intestines,[3] in the saliva of humans,[265, 269] rats[4] and rabbits,[5] in soybeans,[270] in the seeds of many cereals[258–263] and the tubers of plants,[264] in maple sap,[6] cabbage,[7] in wheat,[8, 257] in *Coryne-bacteriam diphtheriae*,[33] in a large number of plants[9] and leaves,[10] such as the mature green leaves of the onion (but not the leek), the snowdrop and the dock,[106] in the eggs of cephalopods,[11] in the organs of silkworms and larvae of butterflies[12] and in human seminal plasma.[13] Certain moulds and fungi produce so much amylase that they constitute an excellent source for its preparation for industrial uses[241–247] (see p. 410), and the same is true for many bacteria[248–256]. This list is neither exhaustive nor significant from the point of view of importance but is given merely to show the ubiquitous nature of amylases in the animal and vegetable kingdoms.

Composition and Site of Activity.—The purification of amylases is difficult, but a number have now been produced in the crystalline state. The impurities accompanying normal preparations of amylases often appear to have a protective action on them

so that the more pure they are obtained the more readily they appear to be inactivated (see below).

The general trend of evidence is that these bodies are proteins and that their activity is not dependent on prosthetic groups.

In some cases broad generalisations as to their composition may be drawn from the action of various compounds on their activity or by the changes in activity brought about by various physical means, e.g. in some cases the amylases are inactivated by heat which might bring about coagulation of protein matter, but with certain amylases heat has a much less marked effect on the activity. H. C. Sherman, M. L. Caldwell and S. E. Doebbeling,[14] for example, prepared a highly active β-amylase preparation which contained 16 per cent. nitrogen and behaved like a typical protein. Denaturing of the protein destroyed its activity. In this instance it is reasonable to suppose that the amylase is either a protein, or that it is a body loosely combined or adsorbed on to a protein without which it cannot exert its full function.

E. Pribam[15] considered malt diastase to consist of two substances, a carbohydrate which only reduces after hydrolysis and a polypeptide containing 7-8 per cent. N. M. W. Beijerinck,[16] in 1908, however, found 'amylase' incapable of replacing either carbohydrate or nitrogenous matter in nutrient media for yeasts and bacteria. H. C. Sherman and A. O. Gettler[35] examined samples of pancreatic and malt amylases and found all of the eight forms of nitrogen distinguishable by Van Slyke's method. Bokorny[36] included takadiastase and malt diastase for total and alkylamide nitrogen estimations, and he considers[37] that the action of pepsin—HCl, shows it to be a protein, while T. B. Osborne[205] considers that malt amylase is a combination of an unknown substance with albumin, the activity corresponding roughly to the amount of the latter.[206] K. Mohs[38] considers that the amyloclastic and saccharogenic amylases differ only in that they are composed of the same protein but differ in their state of 'colloidal distension'. H. Haehn,[39] however, points out that the comparative stability of potato amylase in the presence of proteolytic enzymes indicates that it is not an ordinary protein compound. R. Fricke and P. Kaja,[207-208] furthermore, by electro-osmosis have obtained malt amylases with enhanced amylolytic activity. These gave no protein reactions except Millon's test, but showed a strongly positive carbohydrate test and migrated to the cathode chamber. The important effect of associated protein matter on the activity of amylases is discussed below.

R. A. Kehoe[146] believes ptyalin to be protein in nature, its

activity depending on its combination with certain salts or metals. There are indications that some amylases contain an essential mineral component. The loss of activity of diastase on electrolysis is ascribed by F. Maignon[204] to dissociation of the mineral-organic constituent of the amylase. It is well known that salt-free salivary amylase is inactive. In dialysed saliva a precipitate appears simultaneously with a loss of diastatic activity. On addition of sodium chloride the precipitate redissolves and the activity returns. H. Ninomiya[40] found that the filtrate from the dialysed saliva was inactive but the precipitate can be activated, the greatest activity being shown in 0·05 M NaCl solution, and at *p*H 6·8 with a range of activity from *p*H 4-10. The iso-electric point of salivary amylase is *p*H 4·0 and Ninomiya thus considers it to be in the nature of a globulin. It seems quite apparent that the salt exerts its action by peptising the enzyme, the groups responsible for its adsorption on, and hydrolysis of, the substrate then being exposed and available for work instead of being internally masked, within the unpeptised protein. Purified takadiastase is not activated by NaCl and gives no precipitate on dialysis.

L. Michaelis[41] considers malt diastase to be amphoteric. L. E. Rozenfeld, A. A. Rukhelman and A. A. Zhuravskaya[42] from the reactions of amylase with hydroxylamine and aniline conclude it contains an active aldo group. The possibility of the co-existence of this together with an amphoteric group is not excluded by these workers. E. Rona[43] finds that the activity of amylase is not affected by the presence of substances which react with the aldehydic group. H. Friedenthal[203] has suggested that pancreatic amylase may be a nucleoproteid.

K. V. Giri[44] concludes that the purified amylase of sweet potato is not a protein and has shown, with J. G. Shrikhande,[45] that the enzyme is activated by anions, the effect decreasing in the order F', Cl', SO''_4, NO'_3. However further work has shown that this amylase is protein in nature.

Enzymes occur closely associated with colloids in nature and this may account for the effect of the *p*H value and temperature on the activity, as well as for the effects of some salts which can increase the solubility or peptise proteins.

They are irreversibly inactivated at boiling temperatures, high acid or alkali concentrations, are soluble or highly dispersible in water, dilute salt or ethanol solutions but do not pass through the cellophane membrane of a dialyser. They are precipitated from their solutions in dilute salt or aqueous ethanol by an increase in the concentration of the salt or ethanol and this precipitate can be redissolved in fresh water, etc., to give an active solution.

J. C. E. Weill and M. L. Caldwell[231] find that β-amylase is inactivated by nitrous acid, some 26 per cent. of the activity being lost in 60 minutes, 32 per cent. in 2 hours, and 41 per cent. in 4 hours. They find that neither a first nor a second order reaction will fit the observations. It appears that besides the reaction between the nitrous acid and amino and tyrosine groups of the β-amylase some other groups or reactions are involved, possibly oxidation of SH- or some other essential group, which would account for the sharp initial loss of activity. Treatment with the H_2S may reactivate the β-amylase in the early stages of the deactivation with nitrous acid but after several hours' treatment a slow irreversible inactivation becomes apparent. When treated with ketene β-amylase loses amino nitrogen more rapidly than it is deactivated, 37 per cent. amino nitrogen being lost for a dimunition of 10 per cent. of the activity. From this evidence they consider that free SH- and tyrosine groups are essential to β-amylase activity, whereas free amino groups are probably not. In a second paper[232] they obtained further evidence supporting these conclusions, reactive organic mercurial compounds completely deactivating β-amylase which is reactivated by treatment with H_2S, or cystine, which are without effect on untreated amylase. Iodo-acetamide irreversibly deactivates β-amylase.

These conclusions are in sharp contrast to those drawn from the work on pancreatic amylase.

Thus J. E. Little and M. L. Caldwell,[298, 299] using pancreatic amylase, found that acetylation with ketene leads to rapid decrease in the saccharifying power indicating that this is probably dependent on amino groups since these would be more readily acetylated than the phenolic or sulphydryl groups of the protein. The activity decreases proportionally with the loss of amino nitrogen due to the action of the ketene. Both amyloclastic and saccharogenic activities are affected, the former at a somewhat greater rate than the latter. Other reagents such as formaldehyde, phenyl isocyanate and nitrous acid also yielded results confirming the importance of the amino group and the unimportance of phenolic hydroxy and sulphydryl groups for pancreatic amylase activity. Their work with nitrous acid on the enzyme gives additional evidence that the activity is closely connected with the free primary amino groups of the intact protein molecule. R. L. Lane and R. J. Williams[300] consider inositol to be an active constituent of pancreatic α-amylase.

Crystalline Amylases.—With the preparation by several workers of a number of the amylases in crystalline form, it has been possible to characterise them more closely. M. L. Caldwell, L. E.

Booher and H. C. Sherman[283] reported the isolation of a small amount of crystalline pancreatic amylase in 1931, and were probably the first workers to produce an amylase in the pure crystalline condition. Meyer and co-workers[284–285] have recently prepared crystalline amylase from hog pancreas, from human salivary amylase, from *bacillus subtilis*[287] and from human pancreatic amylase.[288] The crystalline amylases are all proteins but exhibit different crystalline habits, have different solubilities and their optimum *p*H for activity is also different. The bacterial α-amylase differs from the pancreatic amylase also in its affinity for the substrate and the effect that various salts have upon its action.[287] P. Bernfeld and M. Fuld[294] prepared highly pure malt α-amylase and elaborated a rapid and simple method for the determination of α-amylase activity. Using this method they found that the crystalline amylases from swine pancreas, human pancreas, human saliva, *bacilla subtilis* and from fortified malt were remarkably similar in their action upon starch and that the ratio of saccharogenic to dextrinogenic actions were identical. This ratio was found to be six times larger with β-amylase than with α-amylase making it possible to detect β-amylase with an accuracy of 5 per cent.[282] Bernfeld and Studer-Pécha noted a variation between the affinities for the substrate of the different crystalline α-amylases which is responsible for quantitative differences in the course of the hydrolysis reaction particularly after the dextrinisation stage has been passed.[281]

Bernfeld and co-workers[295] found that human pancreas α-amylase as well as salivary amylase gives typical protein colour reactions, coagulates with heat and contains 15·8 per cent. nitrogen and 0·01 per cent. phosphorus. It shows its maximum activity at *p*H 6·9 and is unstable at *p*H values below 4 and above 11. Both of these amylases show an absorption maximum of 2,800 A.U. and a point of inflection at 2,920 A.U. Apart from specific activity, *p*H stability and solubility at *p*H 8 the composition and many physical properties of the human amylase are identical with pig pancreas amylase. It would thus appear that the species and not the organ itself is the deciding factor in the structure of animal amylases.

Crystalline β-amylase from sweet potatoes has been prepared by Balls and co-workers[289–290] and Schwimmer and Balls have also prepared crystalline α-amylase from barley malt.[291, 296] From spectroscopic examination no indications were obtained of the presence of an essential heavy metal or the recognised prosthetic group in the crystalline β-amylase. No α-amylolytic activity was shown by the β-amylase preparation which exhibited its optimum activity between *p*H 4-5. The crystalline α-amylase from barley

malt constitutes the first instance of the crystallisation of an α-amylase from a higher plant, the only other amylase crystallised from a higher plant is the β-amylase prepared by these workers.[296] The α-amylolytic activity of the crystalline product was sixty-seven times as great per unit weight of protein nitrogen than that of the original malt extract.[291]

E. H. Fischer and co-workers[297] have also prepared crystalline malt β-amylase which they found to be a protein containing 14.1 per cent. nitrogen, unstable at its iso-electric point of pH $6 \cdot 0 \pm 0 \cdot 1$, and inactivated by heavy metal ions. This last behaviour is in contrast to sweet potato β-amylase. The molecular weight of crystalline α-amylase from barley was found from osmotic pressure measurements[296] to be 59,500 ± 900, that of a hog pancreas amylase to be somewhat less than 20,000,[285] whilst Danielsson reports a molecular weight of 45,000 for pancreatic amylase and one of 54,000 for malt amylase, calculating the value from diffusion constants and specific volumes obtained from ultra centrifugal studies.

Tauber[314] has described twenty-three different enzymes which are produced by A. oryzae, whilst other workers[312–313] have discussed the various naturally occurring mixture of enzymes.

Myrbäck[312] concluded that barley malt contained, in addition to the known α- and β-amylases another amylase which could hydrolyze limit dextrins to reducing sugars. Kerr and co-workers[307, 308] reported the presence of α-glucosidase in fungal amylase preparations which can convert limit dextrins, whilst dextrinase was the name given by Kneen and co-workers[309–310] to the enzyme in sorghum malt and in barley malt, fungal and bacterial preparations. Corman and Langlykke[306] recognised the importance of a glucogenic enzyme in fungal preparation for the alcoholic fermentation of grain starches, whilst Back, Stark and Scalf[305] and Kneen and Spoerl[311] developed quantitative methods for the estimation of the so-called limit dextrinase.

L. A. Underkofler and D. K. Roy[304] have prepared crystalline α-amylase and limit dextrinase from a filtrate of submerged culture of Aspergillus oryzae. The enzymes were concentrated together by repeated ammonium sulphate precipitations, with a Bentonite adsorption interposed after the first precipitation. The first crystalline precipitate gave two distinctly different types of crystals. The limit dextrinase can be adsorbed on Bentonite after dissolving the ammonium sulphate precipitate in phosphate buffer at pH $4 \cdot 6$. The liquid after treatment gave hexagonal pyramid shaped crystals of α-amylase free from limit dextrinase when salted out by ammonium sulphate.

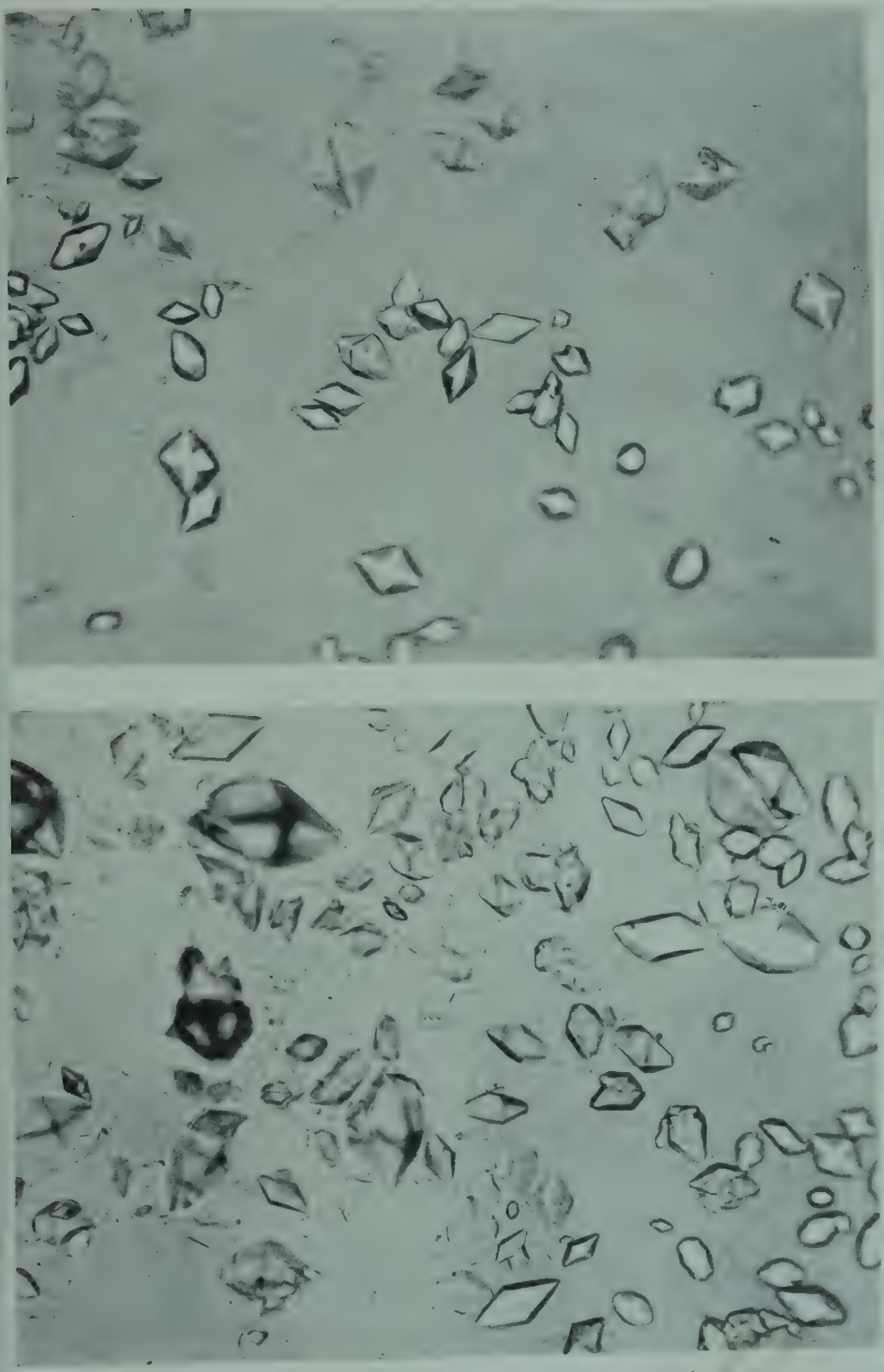

FIG. 18 : 1. Fungal alpha-amylase (x 150).

(*L. A. Underkofler*)

(*Facing p.* 374.

FIG. 18 : 2. Fungal limit dextrinase (x 150).

L. A. Underkofler)

To obtain limit dextrinase in the pure state the original ammonium sulphate precipitate was dissolved in distilled water, dialysed and the solution treated with mercuric chloride which preferentially destroys the α-amylase. Further purification is brought about by dialysing the resulting solution, followed by repeated solution and salting out. The crystals of limit dextrinase are long, shiny, silky needles, and this preparation appears to be the first accomplished of an enzyme of the α-amylase type from a fungal source.

Effect of Temperature and *p*H Value on Activity.—The effects of *p*H value, temperature and the influence of metallic salts and certain organic compounds (see below) may give us some guide to the constitution of the amylases as more data is collected and are certainly of importance in industry. The fact that amylases exhibit an apparent optimum temperature is interesting as it would appear to be the net result of speeding up the reaction and simultaneous deactivation, or partial deactivation, of the enzyme by heat. Thus the *p*H optimum for any amylase will be dependent upon the temperature and the duration of the reaction. This results from the fact that there is an optimum *p*H for activity and another for maximum stability and in industry the conditions are so chosen as to approach one or other of these optimum sets of conditions according to the end it is set out to achieve. Thus with α-malt amylase its optimum activity at 30° C. is exerted at *p*H 5.0 and if the temperature is increased the *p*H value must also be progressively raised to bring it nearer the *p*H optimum for stability, i.e. *p*H 7, to counteract the heat-inactivation of the enzyme which would otherwise take place. Similarly, where conditions are conducive to instability the longer the reaction time and the closer the *p*H for optimum activity approaches *p*H 7. D. H. Cook[46] finds that below the deactivating temperature the rate of hydrolysis of starch by pancreatic or malt amylase is roughly doubled for every 10° C. increase in temperature. P. Kolbach and G. W. Haase,[47] investigating the starch degradation in cooked mashes, found that for malt amylase the optimum temperature varied with the *p*H value as follows: 55-60° C. at *p*H 6·6; 60-65° C. at *p*H 6·0; 55-60° C. at *p*H 5·1; 50-55° C. at *p*H 4·5, and 45-50° C. at *p*H 4·2 for the liquefying action, but the optimal zones are rather broad and not well defined. Similarly, for the saccharification the values for temperature and *p*H optimum were, respectively, 20-40° C. at *p*H 4-4·5; 50° C. at *p*H 4·4-5; 60-70° C. at *p*H 5·7-6·2.

Their work illustrates what has been said above in as much that, industrially it may be desirable to raise the temperature to obtain

a quicker rate of conversion, a 10° C. rise approximately doubling the reaction rate, but progressive rise of temperature finally results in reaching a point at which further increase in temperature has no apparent influence. At this point removal of the enzyme by heat inactivation just balances the favourable effect of heat on the rate of reaction and higher temperatures lead to a more rapid destruction of the enzyme and thus to a fall in the rate of reaction. The more the temperature is raised the nearer to the optimum *p*H for stability must the *p*H of the solution be adjusted to prevent enzyme destruction. Thus there is no true optimum temperature for amylase action since it must also take into consideration the *p*H value of the solution. The 'optimum temperature' generally given by workers is merely that which, under specified conditions, encompasses the most rapid amylolysis.

H. Le Corvaiser[48] (see also [49]) considers that the mineral content of the ash of various starches affects the action on them of diastase owing to the changes in *p*H value it brings about in unbuffered solutions. If buffered it has no effect. At 60-70° C. the hydrolysis is at a maximum in a *p*H range of 4·5-6·8 and very slight at *p*H 3 or *p*H 7·6, at 45° C. at *p*H 4·7 falling off rapidly at lower *p*H but more slowly at higher *p*H values until *p*H 8, then decreasing rapidly. Various animal amylases were found by J. Munk[50] to show marked differences in optimum *p*H values. Many workers have investigated the effect of *p*H value or acidity on amylase action.[51-62] Sherman and Thomas[63] investigated the action of twenty acids and salts and found the optimum *p*H value for malt amylase to lie between 4·2-4·6 and to be uninfluenced by the acid used.[64] The buffer used exerts some influence on the optimum *p*H value; according to G. A. Ballou and J. M. Luck[65] takadiastase has an optimum *p*H of 5·1 at 30° C. when the salts of aliphatic acids are used as buffers. Using phthalate and citrate buffers the *p*H optimum is at 5·4. The effect of the buffer to give changes in the optimum *p*H on pancreatic diastase has also been confirmed by other workers.[66-68] G. L. Funke[69] puts the optimum *p*H value of takadiastase at between 3·5 and 5·5. Hahn[70] has shown that the maximum flocculation occurs at *p*H 3·63 with malt amylase.

Olson, Burkhart and Dickson[273] have also measured the rate and extent of inactivation of the amylase components of malt extracts over the temperature range 60-70° C., and at *p*H 3·3 and 0° C. The latter conditions rapidly inactivated the α-component whilst little effect was noted on the β-component which, however, was quickly reduced to a fraction of its original activity when the extract was heated to 70° C.

S. A. Barinova[271] found the optimum conditions for maltose production by Aspergillus oryzae amylase acting on potato starch to be 4 per cent. of the enzyme, 30 per cent. starch paste at 60° C. but the *p*H or activity of the preparation is not given. Temperature coefficients of hydrolysis of starches by pig's pancreatic amylase was found by S. I. Pronin[272] to be: rice, 2·23 and 1·86; potato, 2·4 and 1·96; maize, 2·23 and 2·03 over the ranges of 17-27° C. and 27-37° C., respectively.

Further papers on the effect of *p*H value on diastatic activity are given in references,[64, 71–72] and the effect of *p*H value on the maltase component present in takadiastase has been dealt with by A. Compton.[105]

C. S. Hanes and M. Cattle[194] suggested the following as being very close to the optimum conditions: β-malt, *p*H 4·8, 0·015 N acetate buffer; α-malt, *p*H 5·3, 0·015 N acetate buffer; *Aspergillus* amylase, *p*H 4·7, 0·015 N acetate buffer; pancreatic and salivary amylases, *p*H 6·8, 0·005 N phosphate buffer. With the two animal amylases 30 mgm. of sodium chloride per 100 ml. reaction mixture (containing 0·2 per cent. starch) was added, this concentration having been found to produce maximum activation.

V. L. Erlich and G. M. Burkert[279] found wheat and barley malt extracts to be more active when extracted with salt solutions than when distilled water was used, 0·1 N sodium bicarbonate solution giving the greatest effect of some twenty salts examined but 1·0 per cent. papain solution gave the most active extract of any tried.

Nomenclature.—By definition an amylase is an enzyme which acts upon starch to produce cleavage of the molecule. This cleavage may be brought about in different ways by different amylases. Several systems exist for naming the particular amylases and two of them are descriptive of the degradation products rather than of the specific constitutional features of the enzymes. Kuhn[17] found that malt amylase acted on starch to give products showing rising (dextro-rotatory) mutarotation, but with pancreatic amylase and that from *Aspergillus oryzae* a falling mutarotation was observed. Accordingly he classified the two types of amylase in α- (falling) and β- (increasing) according as the products showed these behaviours. It is now clear, however, that β-mutarotation does not, by itself, constitute a definition of the specificity of the β-malt enzyme. The other system makes use of the terms dextrinogenic (α-) and saccharogenic (β-) amylase. The latter was suggested by Ohlsson[73] as the sole reducing product formed by the action of this enzyme on starch is maltose. It

is true that dextrins are also formed, but as this worker pointed out these dextrins must be regarded as the residue of the starch molecule after the maltose has been split off and not as the primary fission product of the starch molecule.

Another method of classifying amylases is according to their source, i.e. from higher plants, from lower plants, from microbes or from animals. All of these may be sub-divided to specify the particular species and, in the case of cereals, still further sub-divided to indicate whether the grain was germinated or malted. It is general these days to prefix the name of individual enzyme preparations composed of one component with the α- or β-classification, α- indicating that it is primarily a liquefying and dextrinising enzyme and β- that its function is predominantly saccharifying. Thus *barley malt, wheat malt or rye malt amylase* indicates the plant from which the material was prepared and the fact that the grains were malted. When separated into the two components the resulting preparation would carry the prefix α- or β-, e.g. β-barley malt amylase, indicating the saccharogenic component of barley malt. With some amylases it is not necessary to have this prefix since the enzyme from such sources is known, or considered at present, to be a single component. Examples of this are the bacterial and animal amylases which are of the α or dextrinogenic type. These are therefore specified by the name of the organism or of the animal from which they are derived and, in the latter case, the portion of the body from which the enzyme was prepared, e.g. pancreas, saliva, urine, blood, etc.

E. Ohlsson[18] later noted that the isolated components of malt amylase, namely the dextrinogenic malt enzyme and the saccharogenic malt enzyme, belonged, respectively, to the α- and β-types of Kuhn's classification, the preponderating effect of the saccharogenic enzyme on the mutarotational behaviour of the products leading previously to the false conclusion that malt amylase was solely of the β-type. Other workers have confirmed the dual nature of malt extract, which was originally suggested by Marcker[230] in 1879. Holmbergh,[19] for example, separated them by differential adsorption on rice starch as did Waldschmidt-Leitz and Reichel[20] by adsorption on alumina $C\gamma$ and Freeman and Hopkins[21] reported concordant results when acting on different starch products and glycogen with the two enzymes. The work of the last two workers precludes the possibility that these phenomena are due in either case to mutarotation of unattacked substrate or to those products present in but a slightly degraded form.

T. Chrzaszcz and J. Janicki[91] find that 'sisto-amylases' are present in germinated and ungerminated cereals as well as in

animals. These bodies are presumed to inactivate animal and plant amylases by adsorbing them. The activity is restored by treatment with peptone which they consider acts as an ' eluto-amylase '. This restoration of activity by various means is dealt with below (Inhibition and Activation).

R. Willstätter and M. Rohdewald[92] use the term ' iso-dynamic ' to describe enzymes which act on the same substrate. Iso-dynamic amylases include saccharogenic and dextrinogenic amylases and the ' lyo- ' and ' desmo- ' amylases which are distinguished primarily by solubility. The so-called ' complement ' of amylase is dealt with below.

R. Kuhn postulated two different types of amylase, the α- and β-type, which produce α- and β-maltose, respectively. This, he considered, would indicate either that starch consists of two distinct compounds built up from α- and β-maltose units, respectively, or that both α- and β-forms of glucosidic linkage exist in the starch molecule and on hydrolysis the chain breaks down at one or the other linkage, depending on the type of amylase used. Kuhn's hypothesis is now of historical interest only, as is the similar one of Van Klinkenberg.[26]

G. E. Glock[24] considers starch to be homogeneous, though different varieties or even portions of the same starch appear to differ in their resistance to enzyme action. Samec[25] ascribes this fact to differences in the residual valencies of the molecules of the various fractions, which enable them to combine with varying amounts of the enzyme.

G. G. Freeman and R. H. Hopkins[27] have shown that α-amylase attacks the β-fraction as well as the α-fraction, hydrolysing it completely to maltose (see also C. S. Hanes[28]). The β-amylase is without effect on the α-fraction, which is attacked incompletely by the α-amylase. These workers find that malt α-amylase produces dextrins and maltose but that with β-amylase only maltose and starch appear to be present at the same time. This seems to confirm the theory of Ohlsson, that starch is broken down by α-amylase to dextrin and maltose, but that with β-amylase successive maltose groups are detached. Freeman and Hopkins[29] also found that α-amylase from malt and from pancreas give α-dextrins and then α-maltose, whilst the β-amylases from malt and from ungerminated barley give β-maltose and they confirm that the mutarotation is due to the maltoses formed. J. Blom, A. Bak and B. Braae[30] also consider that α-amylase produces maltose and is responsible for the liquefying action, whereas the action of β-amylase is purely saccharogenic. Later, they[31] formed the opinion that α-amylase acts on potato starch to give dextrins but

that maltose makes its appearance when the reducing power of the medium reaches 7 per cent.

One anomalous fact has not so far been explained, viz. why the action of α- and β-amylases on the same material (some 60 per cent. of the starch) produces α- and β-maltose, respectively.

Another property of certain amylases has not yet been mentioned, and this is the capacity to liquefy starch pastes. The mechanism of this action, which has wide industrial application, is not yet clear but possibly involves a reduction of starch micelles to monomolecules after which dextrinisation and saccharification can proceed. In the first stage neither of these reactions appear to occur, no appreciable production of reducing groups being detectable and the iodine reaction remaining unchanged. A further fall in viscosity will, of course, occur during the subsequent dextrinisation or saccharification of the monomolecularly dispersed starch. Starch liquefying properties are typical of the α-amylases.

The terminology of the products of β-amylase hydrolysis can be confusing. A starch solution is hydrolysed by β-amylase until some 50-60 per cent. is converted into maltose and a portion remains which is *relatively* resistant to further action by the enzyme. The resistant portion is referred to variously as erythrogranulose,[190] α-amylodextrin,[191] α-starch[192] and β-dextrin.[193] Erythrogranulose and α-amylodextrin are the two most commonly used names, although the former is a misnomer, since the material gives a blue colour with iodine.

The Nature and Significance of the Mutarotating Products.—It may be taken as established that the mutarotation in the case of β-amylase is due to the liberation of β-maltose, and Freeman and Hopkins[21] have shown that the rate of mutarotation agrees closely with that of pure maltose under similar conditions. With α-amylases, however, the crediting of the mutarotation solely to the maltose is misleading in that we might assume from this that the reducing products are entirely maltose. Freeman and Hopkins have refuted this and shown that reducing dextrins account for the bulk of the reducing power and but little maltose is present, hence the α-mutarotation is due to groups in the dextrin molecules. With pancreatic amylase, however, they showed that the mutarotation in the early stages is due to the liberation of the α-forms of reducing dextrins with some α-maltose, while α-maltose contributes chiefly to the mutarotation in the later stages.

Thus there is a variation of the proportion of the mutarotating groups attached to the dextrins and to maltose with the amylase

used and the particular stage of degradation, but in special cases the principal mutarotating products are reducing dextrins.

In the case of the α-type, such as pancreatic, *Aspergillus* and α-malt amylases, the preliminary degradation of the starch into dextrin molecules takes place. The liberation of α-mutarotating products therefore appears related to this most common type of degradation.

For some time the only known example of Kuhn's β-amylase type appeared to be the β-amylase of malt and barley which liberates β-maltose by the cleavage, however, of an α-glucosidic linkage (as it cannot now be admitted that β-linkages exist in starch). It would therefore appear that it is the enzyme and the mode of fission that determines the fission product and not the configuration of the starting product. Ugrumov[74] considered, with others, that the appearance of active β-amylase alone in dormant cereal grains depended on the differential inactivation of the dextrinogenic component during maturation of the seed. Giri[75–77] has shown, however, that the amylase of the sweet-potato tuber, Ipomea batatas, induces a closely similar degradation of starch. It appears to contain no α-component and it would appear that this enzyme exists in a state of isolation in the tuber and is closely similar to β-malt amylase.

Amylase Action on Starch.—Native starch is scarcely affected by amylases, and if all the granules were intact it is probable that no action at all would occur with certain starches, e.g. potato. In the plant tissue the erosion of the starch appears confined to localised areas on the surface of the granule, and during the late stages of endosperm depletion in germinating barley numerous empty sacs may be seen. As noted by Brown and Heron[22] these sacs are usually perforated with small holes through which entrance of the enzyme and removal of the fission products of hydrolysis has apparently taken place. These workers consider that these sacs consist of ' starch cellulose ', and Ling and Mehta[23] mention that starch granules are apparently enclosed in a thin membrane that appears to differ decidedly in properties from the starch substance it encloses. The recently postulated ' oxygen bridges ' between the crystals of starch in the granule may, however, give an alternative explanation as to why native starch does not swell in cold water and remains unaffected by amylase but does not seem capable of explaining away the sacs left in the plant as mentioned above. Sande-Bakhuyzen[31] considers it to be a less hydrated form of starch (see also Schoen[32]).

Starch paste which has been stirred at high speed (see Vol. II) or autoclaved at 120° C. for 30 minutes is a suitable substrate

for studying amylase action on starch, but solutions of the so-called soluble starch are most widely used for this purpose. The criteria of starch degradation by the liquefying, dextrinogenic and saccharogenic amylases are discussed in Chapter 24.

By the use of diastatic methods Ivanoff, Kurgatnikov and Kirsanova[172] reported that fine differences were shown by individual starches. The variations in the digestibility of starches from different sources have been noted by W. Stone,[173] J. O'Sullivan,[174] Y. Nagao,[175] H. C. Sherman and co-workers,[180] K. Amberger,[177] A. J. Hermano and O. S. Rask,[178] G. Kashiwaya[179] and others.[218] Nikolaev[217] finds that different varieties of potato starch are saccharified at different rates and to different degrees by diastase, and in such cases the starch pastes had different viscosities. Most investigators report potato starch to be more rapidly digested than cereal starches.

Besides acting on pasted starch α- and β-amylases will act on starch in which the structure has been destroyed by, for example, milling in a ball[181] or rod mill.[176] The effect of pre-treatment temperature on the extent of digestion by diastase has been discussed by E. Wiegel,[182] whilst C. E. Mangels,[183] J. S. Andrews and C. H. Bailey,[184] S. R. Snider and D. A. Coleman,[185] H. R. Sallans and J. A. Anderson,[186] S. Redfern and W. R. Johnston[187] and others have investigated the effect of the substrate on the course of amylolysis. V. D. Martin and J. M. Newton[188] have shown that the temperature at which the starch substrate is prepared affects the rate as well as the degree of digestibility by β-amylase from soybeans. Using soybean amylase the following temperatures of gelatinisation were found to give the maximum rates of digestion of the various starches: potato, 70° C.; tapioca, 80° C.; wheat and corn, 90° C.; rice, 80° C. or above. The yield of maltose was plotted against the temperature of pre-treatment and characteristic curves obtained which differed in their extrapolated intercept on the temperature axis, in their slope and in their value for maximum digestion. These curves are striking enough to suggest the application of this method to the examination of industrially modified starches.[189] Newton and co-workers[189] find that the intercept on the digestion curve, i.e. the point at which digestion by soybean β-amylase commences, closely corresponds with the point at which the polarisation cross begins to disappear and the granules will commence to take up Congo red. They also found that the so-called 'chlorinated', i.e. oxidised with hypochlorite, maize starches were not attacked by malt extract or by soybean β-amylase if more than 0·1 equivalent of chlorine per glucose unit had been employed. They therefore consider

that oxidation leads to alteration of some of the glucose units of starch to structures which are not attacked by β-amylase under their conditions. Dextrinised and acid-hydrolysed starches were also examined, and whereas increased dextrinisation increased the susceptibility at low pre-treatment temperatures and decreased digestion at high pre-treatment temperatures, slight acid hydrolysis generally increases digestibility at high pre-treatment temperatures but with little effect at low pre-treatment temperatures. All this work shows how very many factors can influence the course of amylase action and the necessity of stating all conditions in order to obtain correlation of results with those of other workers. Discrepancies in the literature may be due, in view of Martin and Newton's work, to differences in the method of preparing the starch substrates. E. D. Day[219] is another worker who has studied the effect of cooking different starches on their digestibility by amylase.

Inhibition and Activation of Amylases.—A number of factors appear to influence the activity of amylase preparations and besides the factors of *p*H value of the solution and the temperature, already discussed, the presence or absence of various salts, protein degradation products, lipids and other compounds may variously affect the rate of reaction. It would appear, from consideration of the mass of evidence available, that the 'activators' function by either increasing the stability of the enzyme to external influences or by increasing its solubility.

There is a parallelism between amylolysis and most other enzyme reactions in that it comes to a halt before reaching the theoretical end-point. Many workers conclude that the fission products of the reaction inhibit the enzyme's action. This is considered by these workers to be accountable if one assumes that the enzyme has an affinity for the fission products, which increase in amount as the reaction proceeds. In the case of starch hydrolysis, however, other causes must exist as some of the bodies produced have no affinity for the enzyme (see below[87]).

Holmbergh[79] has shown that if the fission products are separated from the reaction mass, by dialysis or by precipitating them as osazones, the reaction starts again. Effront and also H. van Laer[80] have shown that the amylase can be recovered unchanged at the end of the reaction, and Glimm and Sommer[81] claimed a 100 per cent. recovery. E. R. Moritz and T. A. Glendinning[82] have pointed out that the addition of more starch to the mixture after the reaction has ceased causes it to restart. Brown and Heron[83] and also Kjeldahl[84] considered that the fission products

did not halt the reaction, but in the view of A. Wohl and E. Glimm[85] even small concentrations cause inhibition, and no reversion of these products takes place. Lüers and Wasmund[86] and K. Sjöberg and E. Eriksson[87] have also worked on this subject. Glucose, maltose, dextrin, galactose and mannose have an inhibiting effect, whereas sucrose and fructose are inert. The latter workers consider that pancreatic amylase is inhibited by maltose to a greater extent than is malt amylase and that the action of germinating barley extract on amylose, amylopectin and soluble starch is inhibited to the same extent in each case by the addition of maltose.

R. Kuhn[88] considers that the configuration of the added sugar has a decided influence on the inhibiting effect exercised by the sugar. In general he found that hydrolysis with malt was little influenced by α-glucose but strongly by β-glucose, α-β-maltose and β-maltose. Pancreatic amylase is equally, but only slightly, inhibited by both forms of glucose, whilst α-β-maltose and β-maltose also inhibit equally and more strongly. With taka-diastase α- and β-glucose are equally retarding, but ' equilibrium ' maltose has a greater effect than the β-form. In further experiments Kuhn obtained parallel results when using amylose and amylopectin instead of starch. K. Sjöberg has shown in these cases that the final equilibrium is attained even although the reaction has been slowed down. He further points out that using dialysed malt extract on 1 per cent. solution of soluble starch a 60 per cent. saccharification is attained before the reaction halts and this is not affected by removal of the reaction products by dialysis. Therefore there must be some cause for the halting of the reaction other than the affinity of the enzyme for the fission products.

Inactivation of barley-malt diastase has been brought about by shaking, as shown by Holmbergh,[89] who gives convenient methods for preparing large amounts of pure (α- and β-) malt amylases.[90] L. Hollander[93] finds that rat-liver amylase preparations vary greatly in their ability to digest starch to a blue-violet end-point. Incubation of the aqueous liver suspensions was found to increase the maltose-forming ability and this worker considers that two amylases are present together with an unstable inhibitory substance which can be precipitated by acetic acid.

C. Wunderly[94] discusses whether one enzyme reaction can be influenced by another taking place in the same medium. Casein inhibits the action of salivary amylase on starch, but if trypsin is added the reaction commences. By the addition of more trypsin an activity was shown by the salivary amylase which

was greater than that of the control, and the *p*H optimum for the amylolysis appears to depend upon the *p*H optimum of the trypsin and not that of the amylase. T. Chrzaszcz and J. Janicki[95] find that rennin increases the diastatic activity of ungerminated grain. They find[96] that active papain also increases the amylolytic power of barley and other grains with the exception of millet, which becomes less active. They consider the effect on barley is either due to the production of more amylase or to the setting free of amylase bound in an inactive form to protein. K. Myrbäck and B. Örtenblad[97] consider the β-amylase in barley is partly bound to an insoluble protein and is inactive in this form. By the action of proteolytic enzymes, e.g. papain, the amylase is freed and thus activated. Y. P. Barmenkov[209] finds free amylase to be electropositive (see also Ref.[213]), but in the presence of electro-negative mucin the whole complex may be predominantly electronegative. With amylases which are activated by sodium chloride he considers that the latter breaks up the mucin-amylase process, giving an electropositive NaCl-amylase complex which can exert its fully amylolytic action.

Hydrogen sulphide has been considered by Chrzaszcz and co-workers to activate amylase, but K. Myrbäck and Örtenblad[97] suggest that the amylase had been inactivated in the first place by traces of copper impurities which are removed by the hydrogen sulphide giving the active enzyme. J. Janicki[98] supports this view but maintains that ascorbic acid plays an important role in inhibiting barley amylase. C. S. Hanes[99] has also studied the reversible inhibition of β-malt amylase by ascorbic acid and the allied compounds, reductone and dihydroxy maleic acid. He considers the inhibition is due to the dienol grouping common to these compounds and finds the inhibition is reversed (*a*) by oxidative destruction of the dienol group, and (*b*) by the presence of reducing substances such as hydrogen cyanide, sodium thiosulphate, cystine, thiosalicylic and thiolacetic acids. Methylene blue and potassium ferricyanide reverse the inhibiting effect of ascorbic acid. Ferrous sulphate has but little effect on inhibition, but traces of copper increase the inhibition.

J. Janicki[98] also ascribed the inhibition of amylase in the presence of ascorbic acid and its re-activation by other substances to oxido-reduction processes. He considers the inhibition to be associated with the dehydrogenation of the ascorbic acid. It is reversible and can be prevented by hydrogen sulphide and other reducing substances. The dehydrogenation of ascorbic acid and therefore the inhibition of amylase is strongly accelerated by traces of copper, so that it is important to use carefully purified

water. This dehydrogenation process also takes place in starch-buffer solutions. If the re-oxidation is accelerated by the addition of water containing traces of copper then the reversibility of the activation brought about by the hydrogen sulphide can be demonstrated. Ascorbic acid also inhibits α-amylase. This inhibition results from an oxidation of ascorbic acid and can be prevented by hydrogen sulphide. The disappearance of the α-amylase at the end of the ripening period of barley cannot be ascribed entirely to the oxidation of ascorbic acid since hydrogen sulphide does not, in this case, restore the dextrinogenic and liquefying power of the amylase. R. Weidenhagen and P. Lu[100] find the inhibition by ascorbic acid is abolished by thiol and S—S compounds, amino-acids and citrate.

C. P. Deshagirirao and K. V. Giri[275] have confirmed Janicki's findings and have also showed that substances which inhibit the catalytic oxidation of vitamin C, or ascorbic acid, by copper such as oxalic acid, uric acid, xanthine, xuanine, yeast nucleic acid, creatinine, cysteine, cystine, glutathione, histidine, potassium cyanide and hydrogen sulphide annulled the inhibition and the inactivation caused by ascorbic acid, ascorbic acid-copper complex, and by the oxidation products of ascorbic acid. Pao Chung Lu[276] notes that 62 per cent. of the activity of β-amylase, acting on rice starch, is destroyed by the addition of as little as 0·001 mol. of vitamin B_1 to 37 ml. of reaction mixture.

Chrzaszcz and Janicki[91, 228] have described the inhibitory nature of an insoluble substance present in buckwheat and Kneen and co-workers[229] have discussed the water-soluble, protein-like compounds which are present in wheat, rye and the water-soluble, high molecular, organic acid present in certain sorghums, which appear to be true inhibitors since their action is specific against certain amylases and not against others. The actual inhibition here appears to be due to adsorption since the adsorption complex can be broken up to liberate the amylase with its properties restored. The inhibitor from wheat is definitely a protein[235] and its mode of action is non-competitive.[236]

Unlike certain inorganic poisons these inhibitors show a certain specificity in their action which has suggested to F. J. Di Carlo and S. Redfern[237] and to E. Kneen and L. D. Beckord[238] the possibility of their being used to differentiate and identify the amylases. The inhibitor from wheat affects all animal and one of the bacterial amylases and that from one of the sorghums inhibits all amylases except those from fungi. H. C. Eyster[239] found that indole-propionic acid inhibited the action of the amylases and it is of interest that the indole nucleus has been

found in both the wheat and the sorghum inhibitors. Di Carlo and Redfern[278] have also examined a number of metallic salts and organic compounds for their inhibiting action on B. subtilis α-amylase. From their results they consider they have established the presence of essential SH groups and that the presence of essential amino groups is also indicated (see also Ref. 315).

The mode of inactivation of β-amylase in the barley grain is discussed by K. Myrbäck and S. Myrbäck[101] and by Chrzaszcz and Janicki.[102] The former workers consider that the action of hydrogen sulphide or cyanide is to activate the barley proteases, which in turn liberate the amylase in an active form (*vide supra*), but Chrzaszcz and Janicki consider proteolysis is of no importance in the activating cycle and point out that no parallelism is found between the rate of activation and the rate of proteolysis. K. Myrbäck and S. Myrbäck consider that this argument does not refute their postulate, but the demonstration of such a parallelism would be the only convincing proof of their hypothesis. As we have seen above, it appears fairly certain that proteolysis may play some part, for instance, in liberating the amylase in a soluble, active form, but another more direct activation would appear to take place as well. All workers agree that only the β-amylase of barley is activated. It is interesting to note that H. C. Gore and C. N. Frey propose to add protein matter, to the extent of 1 per cent. on the weight of starch to be converted, together with a buffer, such as sodium acetate, to enzymatic starch conversions to activate the enzyme. They suggest that the proteins thus added combine with the tannins present in the mash material and prevent them from combining with, and inactivating, the amylase. According to K. V. Giri and A. Sreenivasan[103] and Chrzaszcz and Janicki[104] in the early stages of the ripening of the grain, rice, barley, wheat, rye and oats, α-amylase is present but becomes inactive as the ripening proceeds and then regains its activity during sprouting.

Many of these workers have studied the nature of inactive amylase in barley and similar materials and great variations appear to exist in barleys grown under different conditions.[111, 112, 277] The distribution of amylase in the barley kernel has been studied by K. Linderström-Lang and C. Engel[113] and others,[114] while several workers[115–116, 315] have dealt with the fate of the amylases during the ripening and germination.

Amylase-Complement, or Co-enzyme, and Organic Activators.—Pringsheim and co-workers, using pancreatic and salivary amylases, found certain substances, 'amylase-complement', increased the activity. Certain yeast preparations,[120] egg-

white, blood- and serum-albumin which have been digested with pepsin, were found to be good activators.[121–123] The egg-white complement is heat-labile but that from yeast is fully resistant to heat. O. Holmbergh[79] activated pancreatic amylase with living yeast and obtained complete saccharification, but dead yeast extract was without effect. Bakers' or brewers' yeast treated for a short time with toluene retained its maltase action. As the maltase and the pancreatic amylase possess the same optimum *p*H the presence of the former in the yeast will affect the results. In the case of malt amylase no complementary reaction is observed although the maltase in this case would not be operative owing to the malt amylase having its optimum *p*H value on the acid side.

However, K. Sjöberg,[124] R. Kuhn,[17] J. Blom, Bak and Braae have found the complement from yeast greatly increased the hydrolysis of starch by malt. Amylose, amylopectin, soluble and ordinary starch were found to be more vigorously acted upon by malt with the yeast complement present, although Sjöberg found one of Pringsheim's complement preparations to be inactive. L. de Hoop and J. A. van Laer[125] consider it possible to get an almost quantitative yield of maltose if an amylase rich in co-enzyme or complement is used.

R. Weidenhagen and A. Wolf[126, 127] dispute the existence of amylase-complements, but Pringsheim, H. Borchardt and H. Hupfer[128] maintain their previous claims. They have further shown that glutathione, which is contained in autolysed yeast, acts as an amylolytic activator.[129–131] E. W. Rockwood[132] has studied the stimulating effect of glycine and aspartic acid on the action of ptyalin and introduces the term 'auxo-amylase' to describe amylase-complements or co-enzymes[141]. The action of pancreatin is speeded up by sodium taurocholate and glycocholate,[134, 167] and other workers have noted the stimulating influence on amylases of amino-acids,[133, 135, 140–142, 147–149 303] certain albumins,[136, 137] and bile[138, 145, 171]—in which the activator is thermostabile, dialysable and alcohol soluble, but which G. Buglia[139] considers merely lowers the surface tension—and asparagine or aspartic acid[143] whose effects are interchangeable rather than additive in admixture. Lysine has no effect on the amyloclastic activity of purified pancreatic amylase but favourably influences its saccharogenic action.[149] Tryptophane has also been shown to have a similar effect on this amylase and also on salivary amylase.[150] Caffeine is without action[301] but coffee extract has been found to increase the rate of digestion of starch by salivary and pancreatic amylases.[302] Sherman and his co-workers[149] consider pancreatic amylase to be a protein and

suggest that lysine in the enzyme molecule is not split off until the stage of amyloclastic action has been past.

It would appear that activators may act by several different mechanisms among which may be suggested (*a*) increasing the stability of the amylase to external influences either by direct combination with it, or by combining with the interfering agent, (*b*) by increasing the solubility of the amylase. Pigman[220] and Kneen and co-workers[225] (*vide infra*) have shown that calcium ions to serve no other purpose than to stabilise α-malt amylase having influence on the rate of reaction in the early stages but reducing the rate of loss of the enzyme's activity.

W. W. Pigman[220] has studied the extent to which the enzymic conversion of maize, waxy maize and potato starches, in concentrations of between 10 and 13 per cent., by various amylases into fermentable products is affected by the presence of yeasts. The rather high concentration of starch used in this work was selected as falling in the range employed in the fermentation industries.

When amylases were allowed to act on starch pastes in the presence of yeasts he found complete conversion to fermentable material to occur but not if the amylases and yeast acted separately. He estimated the degree of conversion by determining the alcohol produced by the various mashes and from blank fermentation experiments with maltose and dextrose to determine the fermentation efficiency. From these figures the amount of fermentable sugars produced from the starch can be calculated. Table XXXI shows the results obtained with malt, in the presence of yeast, on a number of starches :

TABLE XXXI

Starch	*Alcohol from 24·96 gm. of dry starch**	*Conversion Efficiency % of theoretical*
Maize	12·78	101·9
Wheat	12·81	102·1
Potato	12·71	101·3
Waxy maize . . .	12·54	100·0
Lintner soluble starch .	12·07	96·2
Theory	(12·54)†	(100)

It is interesting that the amount of alcohol obtained from Lintner starch appears significantly low and it may be that this

* Corrected for malt blank.

† For 88·5 per cent. fermentation efficiency as determined for blank experiments with dextrose and with maltose and calculated from the Gay Lussac equation.

modification produces a small amount of amylase-resistant material.

Cereal α-amylases and the fungal amylases appear to be capable of bringing about complete conversion whereas the cereal β-amylases, certain bacterial and pancreatic amylases convert the starch only partially to fermentable material in the presence of yeasts. This worker considers that β-amylases are not essential for the saccharification of starches to fermentable substances. This would imply that the Lintner method,[221] which mainly evaluates β-amylase activity, is not sufficient for the determination of amylase activity of commercial diastase to be used in fermentation work and that the α-amylase activity value would be the more valuable determination. Amylose prepared by the Schoch butanol-precipitation method is completely hydrolysed by the wheat and soybean β-amylases, which is in agreement with previous work[222-223] With soybean β-amylase there appears to be very little difference in extent of hydrolysis in the presence and in the absence of yeasts.

Although wheat extracts are rich in β-amylase and have been suggested as diastatic agents for the conversion of wheat to fermentable compounds this work indicates that an addition of α-amylase (in which they are low[224]) would be desirable to make them suitable for industrial starch conversions.

When fermentation of the products of amylolysis takes place in the absence of amylases complete conversion to fermentable sugars does not take place. All the enzyme preparations studied by this worker lacked the ability to hydrolyse starches completely in the absence of yeast. He ascribes this to the synthesis of unfermentable material during the diastatic action in the case of the fungal, bacterial and pancreatic preparations, to the formation of a residual high molecular weight dextrin in the case of β-amylases and finally, in the case of cereal β-amylases to an equilibrium established during amylolysis such that about 10 per cent. of unfermentable material is in the equilibrium solution.

Activation by Inorganic Compounds.—Many workers have shown that if salivary or pancreatic or liver amylase is dialysed sodium chloride is removed and the enzymatic activity is lost.[151-160] O. Holmbergh[79] has studied liver amylase in detail and considers in opposition to Kendall and Sherman,[155] Böhne[161] and H. Roger,[163] that the usual buffers are equally good activators and that the phosphate ion has no especially beneficial influence. Willstätter and co-workers[167] considered that the phosphate ion has merely brought the mixture to a very suitable *p*H value and thus apparently produced a good effect. Among the salts usually employed the chlorides are particularly beneficial for saccharifica-

tion and the optimum concentrations are, for NaCl and KCl, 0·008-0·5 N, and for $CaCl_2$, 0·008-0·04. O. Kellner and co-workers[162] noted, however, a retarding effect in the case of the amylolytic action of Koji extract in the presence of salt. The iodide ion has an inhibiting effect on the saccharification by hog-liver amylase, but accelerates dextrin formation, and in the presence of potassium iodide malt amylase shows a decreased, but salivary and pancreatic amylases, an increased saccharifying power.[164]

R. Llamas[233] finds that pancreatic amylase is 40-50 per cent. deactivated by a 90-minute contact with 0·2-0·5 per cent. hydrochloric acid. In the presence of pepsin the inactivation proceeds somewhat further and in any case the deactivation is permanent.

As mentioned above, pancreatic amylase loses its effectiveness on dialysis but regains it on the addition of salt.[165] L. Michaelis and H. Peckstein[166] find that the chloride ion activates this amylase to the greatest extent and this holds good not only at the optimum *p*H value but also, to a greater extent, in weakly alkaline media such as are found in intestinal conditions. Ammonium and magnesium chlorides are nearly as effective as sodium chloride at the optimum *p*H. Bile acids inhibit at the optimum *p*H even in the presence of chloride. The activating effect of organic amino-acids, noted by Sherman and co-workers[143, 147–150] is ascribed by H. Haehn[168, 169] and U. Olsson[170] chiefly to the protective action they exert towards inactivation by temperature.

Pigman[220] has found that calcium iôns are necessary for the complete saccharification of maize with kaffir malt. Kneen and co-workers[225] consider a factor of prime importance bearing on the stability of the amylases is the calcium-ion content of the solutions. Calcium is a *stability* factor for α-amylase and an *instability* factor for β-amylase and they have used this differential stability effect to prepare the two amylases. The maximum inactivation of β-amylase is brought about by the presence of calcium, a *p*H of 6-7 and a temperature of 70° C. Under these conditions 90-100 per cent. of the α-amylase activity is retained. Conversely, the conditions for maximum retention of β-amylase with complete inactivation of α-amylase have been found to be the *absence* of calcium, *p*H 3·0 and a temperature of 30° C.

Amylase Poisons.—Mercuric chloride,[149] copper sulphate, picric acid and formaldehyde[195] were found by Sherman and co-workers to act as poisons for amylase. The observation that pancreatic amylase is inhibited in the presence of liver amylase may prove to be of physiological interest.[196] Malt amylase is poisoned by a number of metallic salts and other compounds, and U. Olsson[197, 198] finds that the poisoning effect takes a certain

amount of time to develop so that there is a progressive decrease in the activity of the amylase in the presence of the poison with time.

The following table shows the action of various poisons with time:

TABLE XXXII

Poison	*Gram-Mols. of Poison per gm. Dry Weight of Enzyme*	*Time in Minutes*	*Percentage Decrease in Activity of Enzyme*
Alanine	$2 \cdot 8 \times 10^{-2}$	30	100
Ammonium molybdate	$3 \cdot 04 \times 10^{-3}$	15, 180, 540, 1320, 3120	6, 6, 22, 38, 67
Hydroxylamine	$7 \cdot 98 \times 10^{-4}$	30, 960, 1380, 2580	8, 65, 67, 83
Iodine	$1 \cdot 39 \times 10^{-7}$	10, 80, 1260	48, 56, 60
Leucine	$5 \cdot 48 \times 10^{-3}$	1560	5
Phenylhydrazine	$4 \cdot 81 \times 10^{-4}$	10, 40, 360	72, 77, 90
Potassium cyanide	$2 \cdot 14 \times 10^{-4}$	10, 1200	33, 58
Semicarbazide	$4 \cdot 45 \times 10^{-5}$	10, 240, 1920	3, 3, 31
Sodium fluoride	$3 \cdot 0 \times 10^{-3}$	300	3
,, sulphite	$9 \cdot 03 \times 10^{-4}$	600	64
,, tungstate	$6 \cdot 03 \times 10^{-4}$	10, 300, 1380	13, 72, 100

According to Holmbergh malt amylase is inhibited by potassium iodide.[164] The inactivation by phosphotungstic and picric acids is probably due to the formation of an insoluble complex between the acid and the basic group in the molecule of the enzyme.[199] Alkaloids[200, 201] and ethyl alcohol up to 50 per cent. on the malt extract have also been found to act as organic poisons. R. S. Potter,[210] contrary to previous reports, finds that the presence of iron, even in concentrations of 850 parts per million, is without effect on malt amylase. He notes a relationship between the toxicity of a metal and its position in the electrochemical series, metals low in this series appearing the most toxic. The injurious effect in the hot-water extract of spent grains on malt amylase he ascribes to the tannins extracted from the husk. The inactivating effect of gall-nut or apple-tannins on diastatic action had been noted previously by G. Warcollier[212] and others.[214]

The following table shows the percentage decrease in activity with certain quick-acting poisons, chiefly metallic salts:

TABLE XXXIII

Poison	*Concentration*	*Percentage Decrease in Activity*
Formaldehyde . . .	$6 \cdot 87 \times 10^{-3}$	50
Iron chloride . . .	$6 \cdot 16 \times 10^{-3}$	11
Mercuric chloride . .	$6 \cdot 04 \times 10^{-8}$	61
Mercurous chloride . .	$2 \cdot 13 \times 10^{-7}$	50
,, nitrate . .	$1 \cdot 78 \times 10^{-7}$	50
Uranyl sulphate . .	$6 \cdot 44 \times 10^{-4}$	57
Zinc sulphate . . .	$8 \cdot 8 \times 10^{-2}$	13

J. A. Smorodinzew and F. E. A. Iliin[215] find salivary amylase is seriously affected by the following salts in the given concentrations: antimony chloride, 0·01-0·00004 N; tartar emetic, 0·18-0·0006 N; potassium and sodium monohydrogen arsenites and sodium monohydrogen arsenate, 0·1-0·0125 N. Pre-treatment of the starch with 0·1 N aluminium chloride renders it electropositive and resistant to amylases, but pre-treatment with sodium chloride is claimed by G. P. Barmenkov[216] to render it somewhat more susceptible to amylase attack.

F. J. Chung and T. H. Chang[274] found aluminium chloride at 200-400 p.p.m. to retard the action of human, undialysed saliva but in dilutions down to 10 p.p.m. it accelerated the action, 100 p.p.m. showing the optimum effect. Similar results were obtained with alum and in both cases acceleration and retardation required higher concentrations of the salts when phosphate ions were present.

The action of light in activating or inhibiting amylase action has been studied by few workers. A. E. Navez and B. T. Rubenstein[202] find that ordinary and plane polarised light show no differences in their action. The speed of hydrolysis was found to be increased in the presence of some fluorescent substances, especially fluorescein. These workers think that a loose enzyme/dyestuff complex is formed which is light sensitive and acts in the same manner as hydrogen peroxide does when exposed to light and oxidises any inhibiting agent present. Whatever may be the mechanism of the reaction the spectral region lying between the absorption and fluorescence bands is especially active. It may be noted that H. Tappeiner[211] found that eosin in sunlight retarded the saccharogenic activity of amylase and that this effect is shown by those compounds only whose light absorption lies in the green or blue portion of the spectrum.

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Chapter 19

PREPARATION OF ENZYMES USED IN THE STARCH INDUSTRY

A very extensive literature concerning the action of enzymes on starch has developed following research on the structure of starch and on enzyme action, and, although few definitive conclusions have as yet been reached, great use is made commercially of the hydrolytic power of certain enzymes on starch.

A brief summary of some of the many methods of preparation of the various enzymes, many of which are used commercially, is presented below for those who are interested in the foodstuffs, adhesive, textile or other starch-using industries.

Table 14 summarises the chief points connected with the enzymes used commercially, or which have commercial possibilities, for the hydrolysis of starch, and we may now consider briefly the preparation of these substances. They may be classed into :

1. Enzymes from higher plants such as cereals, sweet potato or soybean.
2. Enzymes from fungi or moulds.
3. Enzymes from animal juices.
4. Bacterial enzymes.

The cereals may be subdivided into ungerminated grains and malts.

1. Enzymes from Cereals, etc.—Amylase from malt consists of a mixture of separate enzymes which induce distinctive types of starch break-down. The saccharogenic component produces considerable maltose from starch without destroying the iodine coloration properties, whereas the dextrinogenic component progressively destroys the iodine coloration with the liberation of but relatively few reducing groups (see p. 435). Ungerminated barley grain forms the most convenient source of saccharogenic amylase. This is the only enzyme existing in an *active* state in the dormant grain, its occurrence being confined to the mealy endosperm. It is the enzyme of storage, or dormancy, especially in certain cereals. In other cereals it appears

in only minute quantities or may be absent, a state of affairs holding also in most legume seeds with the notable exception of soybean which appears to be a very promising commercial source of this enzyme. Tubers of various plants are also deficient in β-amylase but here, again, a notable exception is provided by sweet potato tubers in which it occurs in liberal quantities. At the onset of germination the dextrinogenic amylase makes its appearance in the aleurone layer, and later in the scutellar epithelium, and from thence diffuses into the endosperm, upon which the starch granules start to corrode.

In addition to these components there appears to exist in ungerminated grain, according to E. Waldschmidt-Leitz and K. Mayer,[38] another amylase, designated amylophosphatase, which causes liquefaction of the starch. It is separated by adsorption, together with the saccharogenic amylase, on to alumina $C\gamma$, after which the adsorbate is removed by elution and the saccharogenic component removed by selective adsorption on kaolin under appropriate conditions.

After barley has been allowed to sprout under controlled conditions of temperature, humidity, etc., it is carefully dried. During the germination three enzymes are generated. One, cytase, is destroyed during the drying process, another is proteolytic, but the third, which, as mentioned above, is really a mixture, is the most important from our point of view and is called ' diastase '. W. Windisch is of the opinion that the high diastatic value of American malts is due to the smallness of the grains used in that country, as he found that the diastatic activity of the extract from small grains was $1\frac{1}{2}$-2 times that obtained from large grains. To obtain the highest yield of diastase, the grains should be dried at the lowest temperature as is practicable. After drying they are ground and macerated with water and, after standing for an hour or so, the liquid is separated and evaporated, under vacuum at a low temperature, to a syrupy consistency. The syrupy consistency is due chiefly to the presence of sugars and dextrins which act as preservatives for the solution. According to W. Windisch and W. Jetter,[1] the diastase-content of cereals decreases in the order : rye, wheat, barley, oats, corn, rice, and potatoes, the last containing enough diastase to liquefy all the starch present in the tuber. Another interesting observation is that of H. von Euler,[2] who found that if barley was allowed to germinate in N/10 disodium phosphate the diastatic extract prepared from it was 2·3 times as active amylolytically as the extract prepared from the same barley germinated in water. The malt extract prepared as above is very widely used in the textile,

adhesive, and foodstuff trades. The biochemistry of malting has been thoroughly dealt with by Hopkins and Krause[65] and those interested in this particular phase of the subject are referred to their book. As already indicated the kind of cereal, its variety and its growth environment all influence the quality of the malt and it appears that a malt of almost any desired α- and β-amylase content can be prepared by judicious selection of raw material and processing conditions.

Special preparations with enhanced activity have been made by a number of workers. For example, a preparation producing 10,000 times its weight of maltose from a 2 per cent. starch paste in 30 minutes at 40° C. at a dilution of 1 in 9,000,000, was prepared by H. C. Sherman and co-workers,[3-4] who dialysed a malt extract of sp. gr. 1·27 against water for 24 hours, separated the solid matter by centrifuging, and after cooling the clear liquid in ice, dissolved 45 gm. of ammonium sulphate in 100 ml. of liquid. A precipitate formed which was separated by centrifuging, and then dissolved in 500-600 ml. of water, and the solution dialysed for 20 hours. The dialysate was concentrated by freezing, fractionally precipitated first by the addition of an equal volume of 99·8 per cent. alcohol, and next by bringing the alcohol content to 65 per cent. by volume by adding more alcohol, and finally adding ether. The first precipitate was separated and discarded, and the final precipitate was collected and carefully dried below 15° C. in a partial vacuum over sulphuric acid, the desiccator being kept in the dark during the drying operation. These preparations have a very high β-amylase activity, judged by the weight of maltose formed, but only a negligible α-amylose activity if judged by the weight of starch hydrolysed to products giving no blue iodine reaction, as determined by a modified Wohlgemuth's method.[11]

The material so obtained appears to be similar in many respects to that discussed by R. Kuhn[7] and O. Holmbergh,[8] but differs in others. M. L. Caldwell and S. F. Doebbeling[10] find that the two types of product present in barley-malt extract, are both deactivated by heating in solution, give positive protein reactions, but are free from carbohydrates. Although they act differently on starch, they both have their maximum effect at p_H values between 4·3 and 4·6. Other optimum values have been given by other workers,[12-18] but, owing to different conditions under which they were obtained, such as differing buffer solutions, they may not be comparable. R. Fricke and P. Kaja[53, 54] have obtained highly active malt amylase preparations by an electro-osmosis technique.

TABLE XXXIV

Preparation	Appearance	Optimum pH	Opt. Temp.	Catalysts	Killing Temp.	Poisons	Strength used Commercially	Advantages
Malt. Extract. Clenbumol.[32] Brimal.[32] Farinol.[32] Balmal.[32] Diastafor.	Thick syrup to thin liquid.	4·5[14, 26] 5·0-6·5[26]	For liquefaction 60-65° C. Saccharification, 50-55° (50-63° C., used commercially).	0·5 per cent. NaCl stabilises and accelerates action.[35] Small amounts of H_2SO_4 or other acids KH_2PO_4	80° C.	Salicylic acid. Zinc chloride. Formaldehyde. Ammonia. Copper salts.	5-8 lb./100 gal.	Price. Ease of control
Fungus or Mould Preparations. Polyzimes and Laund-Aid Takadiastase.	Thin liquids or pale yellow powder.	5·4 4·8[14]	55-57° C.[33]	Sulphates. NaCl. Phosphates.[34] Aluminium salts.[34]	82° C.		1 lb. to 2 lb./100 gal.	Dry preparations stable to storage. Resistant to antiseptics.
Animal Enzymes. Degomma. Novo Fermasol.	Pale buff powder.	4·6-6·8[25] Limits 4-10·0	46° C.	0·25 per cent. NaCl and 0·004 per cent. $Ca(OH)_2$.	55° C. (70° C.)		2·5 lb./100 gal.	Attacks protein as well as starch. No oxidases, hence no risk of yellow stains on fabric. Fairly stable to storage.
Bacterial. Rapidase. Biolase. Superclastase. Rhenania.	Thin liquid.	7-7·5	70° C.	0·5 per cent. NaCl. 0·025 per cent. Na_2CO_3.	93° C.		5-10 lb./100 gal. (Strength employed is dependent on temperature and time of operation.)	Can use at higher temps. and hence get better penetration. Attacks proteins as well as starch. Stores indefinitely. Resistant to alkalis.

H. Lüers and E. Sellner[5] claim to have prepared a malt amylase having an activity of more than twice that of any preparation previously made from malt, to obtain which they employed the method of selective adsorption which has been used by several investigators in this field. These workers point out the importance of keeping all the solutions cold throughout the operations. Aluminium hydroxide adsorbs many enzymes, almost quantitatively,[6] from solution, with the exception of amylase, which is not so strongly adsorbed as are other enzymes. According to M. A. Rakusin,[19] the portion of the amylase that is adsorbed does not give Ostromlevsky's reaction, whereas the unadsorbed fraction does. The adsorption method has been further studied by H. Kraut and E. Wenzel,[20] who used pancreas extract and plotted the equilibrium curves for adsorbed and unadsorbed enzyme at different concentrations, and in the presence of different amounts of adsorbents. The preparation of pure β-amylase[36] from sweet potato ' malt ' has been described by K. V. Giri.[37] Kaffir malt, from germinated sorghum vulgare, however, contains only an α-amylose.[58]

For industrial uses dry malt may appear as a ground product or a milled flour, and the syrupy concentrated extract of malt is widely used. When the α-amylase activity is a desirable feature the best preparations are probably those in which the enzymes have been precipitated from the malt extract by means of neutral salts or alcohol and dried, since the α-amylase activity is likely to fall to a very low figure in malt syrups due to the instability of α-amylase, especially when calcium salts are present in insufficient quantities for the stabilisation of this enzyme (see p. 389).

Preparation of Individual Malt Enzymes.—When it is desired to prepare the component enzymes of malt comparatively free from contamination by one another three methods are open to us. The principles they embrace are briefly :

(*a*) Differential adsorption, which has already been mentioned.
(*b*) Differential diffusion.
(*c*) Differential inactivation.

Should pure β-amylase be required one may start with the β-amylase-rich, but α-amylase deficient preparations obtained from barley, rye, wheat, sweet potatoes or soybeans which are extracted with dilute neutral salt solutions, such as 1 per cent. sodium chloride. In the case of cereals it is most useful to use papain as an adjunct to extraction. These preparations may then be further concentrated and purified by the following methods :

(*a*) Waldschmidt-Leitz, M. Reichel and A. Purr[39] adjust the malt solution to *p*H 3·8 and adsorb the saccharogenic amylase (with a little of the dextrinogenic amylase) on to alumina Cγ leaving a solution rich in dextrinogenic amylase. O. Holmbergh[40] prepares the latter by adsorbing it from a 40 per cent. aqueous alcoholic solution of the mixed amylases, adjusted to a *p*H value of between 4·4 and 7·6, on to native potato or rice-starch granules at a low temperature. On washing the starch granules with a 1 per cent. solution of maltose in 50 per cent. aqueous alcohol the amylase is removed in a reasonably pure form.

(*b*) H. P. Wijsman[41] and later G. A. van Klinkenberg[27] placed drops of malt extract on a gelatine-starch gel and 3 or 4 days later stained the gels with iodine. There was a colourless central zone up to the limits of diffusion of the dextrinogenic amylase surrounded by a wider violet band showing the extent of diffusion of the saccharogenic amylase. This technique has been used by Giri (see Vol. II) to distinguish between samples of pasted starch. By repeating the above experiment under the same conditions, omitting the iodine staining and removing small blocks of the gel where the outer zone would have been made visible if stained by iodine, and bringing these blocks into contact with a new gel, it was found that only mauve diffusion zones were obtained on staining. This showed that the saccharogenic amylase had been separated entirely by diffusion. Using this technique K. V. Giri,[42] without the confirmation furnished by the secondary diffusion, concluded that *aspergillus* and pancreatic enzymes were mixtures.

(*c*) While Wijsman's work foreshadowed the later methods of differential inactivation by weak acids and heating, respectively, E. C. Ohlsson[12] provided reliable methods for preparing relatively pure preparations of the component enzymes of malt. The saccharogenic enzyme is rapidly inactivated at elevated temperatures, but is not deactivated by acid. The malt extract at 0° C. is therefore adjusted to a *p*H value of 3·3 with hydrochloric acid, whereupon the dextrinogenic activity falls to 1-2 per cent. of its original value, whereas the drop in saccharogenic activity is only of the order of 20-30 per cent. The dextrinogenic enzyme is unusually thermostabile so that a malt extract of *p*H value 6-7 retains 60 per cent. of its dextrinogenic activity if heated to 70° C. for 10 minutes, whereas the saccharogenic activity has fallen to 5-7 per cent. of its original value.

Blom, Bak and Braae used the differential inactivation method to prepare the purified α- and β-malt amylases. Their conditions were less drastic than those used by Ohlsson (*vide supra*).

They found that acidification of malt amylase preparations at 20° C. and *p*H 4·2 resulted in almost complete destruction of the α-amylase in 4 hours whilst heating the neutral enzyme solution to 60° C. for 1 hour inactivitated the β-amylase completely.

E. Waldschmidt-Leitz[43] and T. Sabalitschka and R. Weidlich[44] think that these treatments may substantially alter the properties of the enzymes present in malt, so that the same enzyme could show one of two sets of properties according to the method of treatment, but other work has shown beyond doubt that two amylolytic enzymes do exist in malt extract.

Von Klinkenberg[27] finds that a better yield of β-amylase is obtained from wheat than from barley. Hanes[59] has described a simple method of separating β-amylase which has been used by Haworth and co-workers[60] to obtain preparations for use in elucidating the structure of α-amylodextrin. The latter workers extracted ungerminated wheat flour twice with 50 per cent. aqueous alcohol and the enzyme was precipitated from the clear extract by increasing the alcohol content to 80 per cent. The precipitate, separated on the centrifuge and freed from alcohol by suction, was dissolved in water (450 c.c. for the extract from 200 gm. of flour) and filtered. The clear solution contained the β-amylase and gave a negative Wijsman test[41] (*vide supra*) for α-amylase.

The preparation of soybean β-amylase has been described by Newton and Naylor[62] and by Pigman.[61] Palmetto and Missoy varieties of soybeans were ground, defatted with acetone and 800 gm. of the defatted material treated with 3,200 ml. of 50 per cent. ethyl alcohol containing 1·6 gm. of sodium sulphite, the *p*H of the solution being adjusted with acetic acid to 6-7. The mixture is treated for 3 hours at room temperature with frequent shaking, filtered under suction and the residue washed with about 500 c.c. of 50 per cent. ethanol, the washings being added to the original filtrate. The extract is cooled in ice to 0° C. and 1,870 ml. of 95 per cent. ethanol added slowly with mechanical stirring. After allowing the mixture to settle for 15-30 mins. the supernatent liquid is poured off and discarded. The gummy precipitate is covered for several minutes with acetone which is then poured off, this process being repeated several times after which the gummy material can be readily powdered with a spatula. The powder, after removal of the acetone is stored over calcium chloride in a refrigerator. The yield is 0·9-1·2 per cent. the weight of the defatted bean flour, and the activity of these preparations are reported to be between 2,400-3,400 I.[63]

2. Enzymes from Moulds or Fungi.—Moulds have been

used in Japan for centuries to prepare alcoholic beverages from starch-bearing materials and Atkinson appears to have been the first writer to publish a paper in English on this reaction. In his paper he noted the sensitivity to heat of the amylase of 'Koji'. Certain species of the yellow-green mould *Aspergillus oryzae* produce a mixture of enzymes, including diastase, invertase, maltase, amidase, and protease, the amounts of which vary with the different species employed. To obtain a preponderance of any one enzyme, it is therefore necessary to select the desired species and to isolate it in the pure state by repeated subculturing. The pure culture so obtained is immunised against certain antiseptics that are met with in the textile industry (if the preparation is intended for use in desizing operations), by subculturing it on a semi-solid nutrient media containing increasing amounts of the selected preservatives, e.g. sodium fluoride and salicylate,[31] until a strain is finally obtained which possesses a marked resistance to the action of these compounds. This strain is then used as the starting-material for preparing batches of the enzyme preparation on a large scale. A mass of sterilised nutrient material consisting of wheat bran,[71] corn, or rye, and containing certain salts, is placed in large rotating drums kept at 30-40° C. and adjusted to *p*H 4·0 to prevent bacterial contamination, and is inoculated with the culture. After 2-3 days this has spread over the mass and the enzymes have formed, they are separated by extraction with water, the solution filtered, or separated by centrifuging,[30] standardised to a given strength, preservative added, and the preparation is ready for marketing. Sterile wheat bran appears to be the favoured media for growth of Aspergillus oryzae[72–75] and Underkofler and co-workers[75] have given a full description of the commercial production of mould bran using swinging trays.

The conditions of agitation and aeration are critical for the success of the process and a period of rest under anærobic conditions, to permit rapid growth, followed by vigorous aeration until maximum growth is attained has been suggested.[66, 67] The strength of the hydrochloric acid used is preferably about 0·3 N and not above about 0·45 N.[72]

The preparation of the 'fungi diastase' by submerged culture propagation of Aspergillus oryzae in distiller's thin stillage and the use of the resulting liquor as converting agent in distilleries was suggested by Woolner and Lassloffy[76] and N. M. Erb and co-workers[77–78] and others[81] modified the process. Complete replacement of malt with culture liquors of Aspergillus Niger NRRL 337 produced by the submerged culture processes was first achieved

by Van Lanen and Le Mense.[79] H. M. Tsuchiya and co-workers[80] have examined the effect of composition of the culture medium upon the amylase and maltase production by A. Niger NRRL 337 and 330 and by A. oryzae NRRL 458 when grown in submerged culture.

To obtain powdered preparations of high activity the enzymes are precipitated with alcohol, purified by several treatments, and dried at a low temperature. Sherman and Tanberg[21] have obtained very active preparations from commercial takadiastase in the same manner as they prepared the active malt diastase described above. K. Oshima[28] finds both amylase and protease are obtained simultaneously by the action of *Aspergillus oryzae* on both natural and synthetic culture media. The optimal quantity appears after two days' growth and both enzymes are extra-cellular after sporulation.

Different species of *Aspergillus* show variation in activity of enzyme. T. Nagatomo[29] has noted a deviation of the optimum *p*H value for the amylases from various strains of the fungus. In general the optimum activity is shown between limits of *p*H value 4·8-5·2 and most stable towards heat at *p*H 6·4. L. A. Underkofler and co-workers[46] have examined the saccharification of starchy grain mashes for the alcoholic fermentation industry and find two strains of *Aspergillus oryzae* are eminently suitable. They did not separate the enzyme but used the mouldy bran in the same manner as malt and obtained about 12 per cent. higher alcohol yields than with good barley malt. They describe the preparation of the mouldy bran on a pilot-plant scale[46] and on a 10 ton per day scale.[75]

The commercial preparation of amylolytic enzymes from *Aspergillus oryzae* was first introduced by Takamine,[47, 68] under the name *Takadiastase*, which is often used in the general sense to refer to purified concentrates of fungal amylase. Further descriptions of the preparation and properties of the enzyme from this source are given by L. Wallerstein,[30] Oshima and Church,[49] H. Leopold and H. G. Germann[57] and by Harada.[50]

L. C. Hao and J. M. Jump[82] have fully discussed the many commercial bacterial and mould preparations used to replace malt as a converting agent in pre malting corn mash. Satisfactory liquefaction can be obtained in every case, whether the mould preparations are used in either the crude, the extracted or the precipitated forms.

Wallerstein[83] has described how the amylase from *A. oryzae* and from malt can be completely precipitated by the addition of lignin at *p*H values of 3·6 and 4·5, respectively, and again re-

suspended in active form at *p*H 6·5 or above, a higher degree of recovery of the malt amylase and an increase in its stability to solutions more acid than *p*H 4·5 is obtained, if gelatine is added to the liquor prior to the precipitation with lignin.

3. Enzymes from Animal Juices.—The source from which enzymes are obtained in the animal world are the pancreatic glands of horses, pigs or of cows. The liquids expressed from these glands are rich in starch-converting enzymes which are precipitated from the liquor by salting out. The precipitated enzymes are dried and mixed with neutral salts, which serve to adjust the activity to a desired standard strength, so that the product shall always be uniform in activity when it comes on the market. According to J. Larsen and C. F. Poe[55] aqueous acetamide was found to be the most selective solvent for extracting the amylase from pancreas.

Sherman and Schlesinger[22] prepare an extract of high activity as follows : 20 gm. pancreatin powder are macerated with 200 ml. of 50 per cent. alcohol, and the suspension filtered after 20 minutes. The active constituent is precipitated as an oily mass by pouring the solution into 1,400 ml. of a mixture of 20 per cent. alcohol and 80 per cent. ether, and after decanting the liquid, the precipitate is re-dissolved in a little water, re-precipitated by pouring into five times its volume of absolute alcohol, and the flocculent precipitate obtained dissolved in 200-250 ml. of 50 per cent. alcohol containing 5 gm. maltose. This solution is dialysed three times against ten times its volume of 50 per cent. alcohol for 15, 9, and 40 hours, respectively, for the three portions. The three dialysates are mixed, filtered, and poured into an equal volume of a 1 : 1 alcohol-ether mixture. The precipitate which forms is filtered off and dried under vacuum. The temperature at which the above operations are carried out should be around 10-15° C. throughout, and should never exceed 20° C.

Sherman and Schlesinger found that such preparations, used at a concentration of one in a million on a 1 per cent. starch solution, completely digested the starch to the stage of giving no iodine reaction in 96 hours. In this time the enzyme had produced over 500,000 times its own weight of reducing sugar, and its activity on the Lintner Scale was of the order of 5,000-6,000. Later it was found[23] that only about 5 per cent. of the amylolytic activity was recovered in the final product, and that the precipitate formed in the inner solution during dialysis had a high proteolytic and little or no amylolytic activity.

4. Bacterial Enzymes.—Boidin and Effront[48, 51, 52] may be credited with the first commercial method for preparing amylo-

lytic enzymes by the use of bacteria. The enzymes are of the liquefying type, characterised by maximum activity in alkaline or neutral solutions. Certain micro-organisms of the *B. subtilus* or *mesentericus* species when allowed to act on a sterilised nutrient medium of wort produce powerful diastatic and proteolytic enzymes, and owing to the rapidity of action of preparations made in this way, they have been marketed under the name of Rapidase (see Table XXXIV). This preparation is notable for the high temperatures at which it can work, which in textile work means better penetration. It has been noticed that this preparation does not appear to contain any albuminoid or protein matter that is heat-sensitive, whereas other enzyme preparations from other sources appear to contain such constituents, and this may explain why it is active at temperatures at which other enzymes show diminution, or even destruction, of their activity. Table XXXIV shows the salient features of this preparation.

The bacteria is allowed to grow over large surfaces in thin layers so as to produce a thin film. For maximum liquefying power Boidin and Effront found successive subculturing in a culture medium relatively rich in nitrogenous material was best. Strong aeration at the start followed by a slight lowering of the oxygen supply after full development was beneficial and for most strains of the bacteria used temperatures between 25-30° C. are best.

The culture medium consists of hydrolysed soybean cake or peanut cake, together with hydrolysed starchy material and phosphates, potassium, magnesium and calcium salts. The media is sterilised by heating under pressure at 125-130° C., filtered, cooled and inoculated. The media on trays is kept in a culture apparatus (Fig. 19:2) some 8 feet in height and 6 feet in diameter[41] under a continuous internal pressure, so that air-borne contamination cannot take place and each vessel has a capacity of some 500-1,000 gallons of media. The most scrupulous cleanliness is observed throughout the whole process.

After about one week the liquid is cooled and passed directly to a high-speed centrifuge where the bacteria are removed, after which antiseptics are added to the liquid, which may then be stored. According to the composition, *p*H value and aeration of the media, a large measure of control over the ratio of amylolytic enzymes to proteolytic enzymes is obtained. The liquid can be concentrated *in vacuo* without further processing or, for some purposes, purification by means of precipitation by ammonium sulphate, alcohol, acetone, etc., is carried out. Such processes enable a thousandfold increase in enzymatic activity to be obtained

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FIG. 19:1. Equipment used in microbiological enzyme production.

(*Facing p.* 414.

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FIG. 19: 2. Culture apparatus used in microbiological enzyme production.

Another method of preparation[45] is to take the crude enzymic liquid from the mass obtained by the fermentation of the amylaceous substance with *B. mesentericus* and extract the fatty matter present with a fat solvent. The colloidal matter is precipitated, either by adjustment of the *p*H of the solution or by the addition of heavy metal salts, and the precipitate removed, together with the micro-organisms from the solution by centrifuging. The amylase and protease can then be separated, if required, by the use of various adsorbents. A. Janke and B. Schaefer[56] list 37 species of bacteria that split starch. The most active, according to these workers, were *B. mesentericus*, *B. megathericum*, *B. vulgatus*, *B. mycoides*, *B. subtilis*, *B. ovalecticus*, *B. macerans*, *B. albus*, *Sarcina aeritronuxa* and *Sarcina lutea*. The optimum temperature was 40-50° C. in 1/300 molar sodium chloride solution.

For the preparation of an enzymatically active strain of *B. macerans*, M. Blinc[64] recommends that the bacillus should be grown alternately for 10 days each on meat broth, potato mash and potato slices. After several weeks one can grow it only on potato mash, containing calcium carbonate, at 45° C. and after 2-3 months on pieces of potato tubers 1-4 months old, that have been neither frozen, dried nor damaged. In October or November each year the cultures begin to produce a splendidly growing strain of good enzymatic activity. The activity decreases gradually and in April or May of the following year the culture is poor with only slight enzymatic activity.

To concentrate the enzyme the solution is centrifuged and then filtered through a de Haen's ultra filter, the last portion of the filtrate being the richest in enzyme. Further concentration is obtained by freezing, the last portion to freeze being very rich in enzyme. Fractionally thawing, therefore, gives a very active preparation if the first portion liquefying is taken.

A. A. Imshenetskii[69] and co-workers have studied bacteria which hydrolyse starch at high temperatures. These thermophilic amylolytic bacteria are aerobic, spore-bearing immobile rods which saccharify starch and dextrin rapidly. They require a temperature of 60° C. for growth. The amylase of these thermophilic bacteria is comparatively thermostable, acting on starch most rapidly at 70-100° C. though saccharifying it slowly at these temperatures. The optimal *p*H is 5·5–7·9. The amylase is formed in media devoid of starch, although media containing starch produce a more active enzyme system, more active than *B. mesentericus* in saccharifying starch.

The amylase systems produced by 41 isolates of *Bacillus subtilis* and 7 of *B. polymyxa* and by 3 of *B. macerans* have been

studied by Kneen and Beckord and for details the reader is referred to the original paper.[70]

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Chapter 20

THE ACTION OF β-AMYLASE ON STARCH

Whereas among the α- or dextrinogenic enzymes from different sources there appears to be slight variations in properties or in their action on starch, β-amylase from wheat, barley or other cereals appears to be a definite entity existing in the one form only.[1] It was at one time considered that, as β-amylase existed only in dormant cereal grains, the α-amylase was differentially inactivated during maturation.[2] A number of workers have studied the preparation and properties of amylase concentrates from a variety of cereals such as germinated wheat and rye,[53, 55] germinated maize,[56] germinated oats[54] and soybeans.[57–60] There has been some discussion as to whether two enzymes were present in soybeans or not, and the results of G. L. Teller[61] indicate that, although two amylases are probably present, the soybean amylase is a better sugar producer than any other amylase studied with the exception of that in the sweet potato. Recently Giri[3–5] has shown that the amylase of the sweet potato tuber, Ipomea batatas, resembles β-amylase very closely.

Soluble Starch.—A number of workers have followed the action of β-amylase on starch pastes and soluble starch by means of the *optical rotation* of the medium. H. T. Brown and G. H. Morris[6] claimed there was no agreement between the increase in reducing power and the loss in optical rotation, but Brown and Heron, using different conditions, later obtained a good correlation. H. van Euler and K. Helleberg[7] showed that during hydrolysis of the starch maltose is liberated in the low rotatory form, and R. Kuhn,[8, 9] acting on soluble starch and on amylose with amylases from different sources, arrived at his system of nomenclature from a consideration of the optical properties shown by the various products (see p. 377).

E. Ohlsson[10] was probably the first to recognise clearly that malt saccharogenic amylase is a β-amylase and the malt dextrinogenic amylase is an α-amylase. Kuhn acted on electrodialysed soluble starch with both untreated and dialysed malt extract. From his results it would appear that maltose can be split off in the low rotation form so that a higher degree of fission is recorded if polarimetric methods instead of reducing methods are used to follow the enzymatic action. Ohlsson[11] and G. G.

Freeman and R. H. Hopkins[12] have confirmed these results on all essential points.

By following the *reducing power* of the reaction mixtures later workers showed that, in the β-amylase action on starches, reduction products were formed right at the beginning but that the iodine coloration is only slightly affected. The reaction proceeds relatively quickly until the reducing power reaches a value corresponding to 50-55 per cent. of the theoretical maltose. From this point greater resistance to fission is shown and the reducing value slowly rises to its final level. Table XXXV shows the final hydrolysis limits obtained by various workers on different substrates.

The differences observed may be due to variations in the substrate or its temperature of preparation (see p. 382), or to differences in preparing the enzymes. The limit of production of maltose between 60-70 per cent. was found by Martin, Naylor and Hixon[47] to hold also for corn, wheat, rice and tapioca starches.

Wheat, buckwheat, arrowroot,[19, 25] maize and apple,[21, 26] barley, rice and potato starches all appear to be degraded similarly whether a paste from a solubilised form or the native starch is used, and the limit of hydrolysis does not, according to Hanes[21] and Klinkenberg,[27] appear to be appreciably influenced by variations in the enzyme concentration.

A. Tychowski,[24] using different raw starches in the absence of added buffer salts, found the saccharification limit to be independent of the enzyme concentration, *p*H value of media (4·2-7·9), temperature (20-50° C.) or the presence of glucose, maltose or the other degradation products of starch.

R. H. Hopkins, G. F. Cope and J. W. Green[20] observed a considerable rise in the saccharification limit when living yeast was present in the reaction mixture, and they ascribe this to the effect of an amylase activator (see also p. 389) which they considered may have diffused into solution from the yeast. O. Meyerhoff,[28] however, using an amylase activator from oats, was unable to obtain this increased effect.

A number of investigators report a relatively sharp cessation of hydrolysis at 60-61 per cent. fission, whereas in other cases the reducing power has been found to rise very slowly beyond this point until a 64-67 per cent. fission has been attained. C. S. Hanes[29] reports 59-60 per cent. as the limit when using 0·5-1 per cent. solutions of soluble starch, but with solutions containing 0·2 per cent. values up to 66 per cent. were obtained. The concentration of substrate would, therefore, appear to have a definite influence on the fission limit attained.

TABLE XXXV

Hydrolysis of Starch by β-amylase

Substrate	*Enzyme Used*	*Limits of Hydrolysis*	*Observer*
Lintner soluble starch	Barley extract	60-65	Baker[13]
	Dialysed malt extract	59-61	Sjöberg[14]
Amylose (Gruzewska)	Dialysed barley extract	39	Sjöberg and Eriksson[15]
Amylopectin (,,)	Dialysed barley extract	40	Sjöberg and Eriksson[15]
Potato amylopectin	Alcohol precipitated barley extract	67	Ling and Nanji[16]
Potato starch autoclaved at 130° C. for 10 hours	Barley extract	65	Syniewski[17]
Autoclaved soluble starch	Barley extract	66	Pollak and Tychowski[18]
Lintner soluble starch	Wheat and barley β-amylase	64	Klinkenberg[19]
	Alcohol precipitated barley extract	64-65	Hopkins, Cope and Green[20]
	Alcohol precipitated barley extract	61	Hanes[21]
Lintner soluble starch and Fouard starch	β-amylase prepared by Klinkenberg's method	66	Samec[22]
Soluble starch	β-amylase from barley prepared by Blom's method	60-61	Blom, Bak and and Braae[23]
Potato starch autoclaved as above[17]	β-amylase from—		
	barley	62·6	Tychowski[24]
	wheat	68·6	,,
	rye	62·16	,,
Autoclaved wheat starch	barley	65·4	,,
	wheat	67·2	,,
	rye	64·6	,,
Autoclaved rye starch	barley	63·0	,,
	wheat	64·7	,,
	rye	62·5	,,
Autoclaved barley starch	barley	65·7	,,
	wheat	67·1	,,
	rye	61·5	,,
Autoclaved maize starch	barley	59·1	,,
Autoclaved rice starch	barley	58·7	,,

Blom and co-workers[30] report that at *p*H 3·4 hydrolysis ceases at 53 per cent. conversion, but at *p*H 4·6 and 6·8 the normal limit of 60-61 per cent. is attained. This result may be due to the presence of amylophosphatase and it is very tempting to suggest that some α-amylase may have been present, as this is practically inactivated at *p*H 3·4 and could thus exert no beneficial effect which it might hitherto have exerted.

The *iodine coloration* has also been used to study the effect of β-amylase on starch pastes. Until C. S. Hanes and M. Cattle[31] accurately measured the extinction coefficients of the coloured solutions obtained by adding iodine to portions of the reaction mixture at various stages of the hydrolysis, very few quantitative observations on the iodine coloration had been made, with the exception of the work of Samec[22, 32] and Blom, Bak and Braae.[23, 30] From the beginning of the process the extinction coefficient (E) value decreased over the whole spectral range, the curves showing a general similarity in form to that given by undegraded starch and no shift in the position of the maximum absorption peak takes place.

The extinction coefficient of maximum absorption is approximately proportional to the concentration of the residual dextrin. Actually the residual dextrin gives, after 30 per cent. hydrolysis has occurred, a more intense coloration with iodine than the undegraded starch of equal concentration. During the first phase of hydrolysis the decrease in intensity is smaller than the percentage of maltose formed. Samec and Blinc[33] have confirmed this and find that the iodine coloration of peptised starch is more intense than that of the raw starch.

The only products of β-amylase action on starch are the practically non-reducing, amyloid dextrins which give a blue-violet iodine coloration whilst retaining, in a marked manner, the ability to form molecular aggregates, and maltose (see Baker,[13] Syniewski,[17] Hanes,[21] Freeman and Hopkins,[34] Blom and co-workers[30]).

Thus hydrolysis to the extent of about 60 per cent., retention of the capacity to give a blue-violet or violet colour with iodine, due to the presence of the resistant α-amylodextrin or erythro-granulose and the production of maltose as the sole reducing compound formed is characteristic of β-amylase and differentiates it from other amylases.[1]

1. α-amylodextrin.—The name given to the material left at the end of the degradation of starch by β-amylase stresses its starch-like nature. A certain variability in the properties of this product as obtained by various workers may be ascribed to the method of preparing the β-amylase. Using the enzyme prepared by precipitation with alcohol, which also induces concurrent dehydration, an α-amylodextrin with a high rotation ($[\alpha]_D^{20}$ + 221°) and containing practically all the phosphorus originally present in the starch is obtained.[16] Using an untreated barley extract or a freshly precipitated amylase which has only had a short contact time with the alcohol an α-amylo-

dextrin with no phosphorus and a low rotation ($[\alpha]_D^{20} + 193°$) is obtained.

These results are readily explained if the presence of amylophosphatase (see Chap. 2) in the latter preparation is assumed and that the longer alcoholic treatment in the former case destroys its properties. This would lead to an aggregated, high phosphorus-containing, high rotational but low reducing form of α-amylodextrin from the action of alcohol-treated enzyme preparation. Where no destruction of amylophosphatase had taken place, as in the case of the untreated barley extract, the disaggregated phosphorus-free, lowly rotating product showing a fairly high reducing power should be obtained, as is the case.

Should the presence of amylophosphatase be proved in such cases it further follows that the slow increase in reducing power from 60 per cent. to 66 per cent. apparent conversion may be due to the transformation of the aldehydic end unit of the α-amylodextrin into a reducing form and not to the slow liberation of further maltose.

Samec[22] obtained a low rotating form of α-amylodextrin which gave R_M 16 by the hypoiodate method, which corresponds to a chain-length of some 12 glucose units, which is in agreement with end-group assay results.

The α-amylodextrin, if separated, can be shown to be highly but not completely resistant to β-amylase. By autoclaving, a technique devised by Hopkins and co-workers (see Hanes[35]), further degradation can be brought about, and if the resistant material left after this treatment is successively separated, autoclaved and treated with enzyme a yield of only 3 per cent. of resistant material on the original starch is attained after six such successive cycles.

The α-amylodextrin is not resistant to α-amylase, the iodine coloration being destroyed, and Klinkenberg[19, 25] and Hanes[21] report that β-amylase will further degrade the products of this reaction and that this effect is to be noted from the beginning of the α-amylase action.[21]

2. Amyloamylose.—The degradation of amyloamylose with β-amylase resembles the degradation of soluble starch which can be attained by periodical resort to autoclaving. It should be noted that autoclaving starch is the first step in the process for preparing amyloamylose. This step is followed by electrodialysis in which a gelatinous material, consisting of impure erythroamylose, migrates to the positive membrane. By repeated autoclaving followed by electrodialysis this impure erythroamylose

gives the pure material, which has a red iodine coloration, after some five or six cycles.

It has been variously reported that amylose and amylopectin are hydrolysed at very different rates by amylases. Ling and Nanji[16] reported amylose to be completely hydrolysed by β-amylase or by malt diastase, i.e. α- and β-amylase, and concluded that the amylopectin was not attacked. Van Klinkenberg, elaborating on the resultant definition of amylopectin suggested by the work of Ling and Nanji, proposed the view that selective hydrolysis of a preformed fraction in the starch is responsible for the halt in the degradation by β-amylase at 60-65 per cent. conversion.

Hirst and co-workers[36] were unable to confirm Ling and Nanji's results, finding amylose and amylopectin were hydrolysed to the same extent as starch, and Hanes[39] obtained the same result with amylose and amylopectin prepared by milling starch.

Samec and Waldschmidt-Leitz[37] and, later, Freeman and Hopkins,[38] Samec[22] and Hanes[26] found that the amyloamylose of Samec and Meyer is degraded almost to completion by β-amylase. It may be mentioned here that the discrepancy between the apparent degree of fission given by the polarimetric and the reduction methods noted for soluble starch is also found in the case of amyloamylose. As determined by optical methods the apparent degree of fission passes through a maximum.

Amyloamylose is hydrolysed at a much slower rate than soluble starch, probably because of its higher mean molecular weight, and when about 60-70 per cent. fission is reached the reaction slows down but reducing values corresponding to 95-98 per cent. of theoretical maltose are gradually reached. In this reaction the blue colour given with iodine persists up to R_M 80-90 per cent. and from then on changes through violet to pink.

3. Erythro Bodies.—In contradistinction to amyloamylose, erythroamylose resists further action of β-amylase after some 40-56 per cent. conversion has occurred.[12, 22] These values are slightly lower than the usual limits found for soluble starch but probably not for autoclaved starch paste under similar conditions. Soluble starch and Fouard starch probably contain both amylo and erythro bodies and behave in a manner intermediate between that of amyloamylose and erythroamylose when treated with β-amylase.

4. α- and β-Glucosides.—Blom and Braae[40] have compared the action of amylases with the glucosidases. None of the glucosides are split by α-amylase, and β-amylase does not affect the α-glucosides but splits β-glucosides. R. Weidenhagen and

A. Wolf[41] consider, on the experience gained on the hydrolysis of the oligosaccharides, that a chain of glucose units connected by α-bonds would be attacked by α-glucosidases at all points along its length where there are intact α-bonds and yet starch cannot be hydrolysed by α-glucosidase.

The Mode of Action of β-amylase.—As maltose is the sole reducing agent formed by β-amylase action on starch an end-wise mode of attack must be assumed.[42] In view of the properties of the amyloid dextrin which remains, the maltose must be split off from the non-aldehydic end of the chain-molecule of starch, as any other type of attack would inevitably produce some glucose which, however, has so far not been detected.

Ohlsson[10] found by osmotic pressure data that, during the partial conversion of the substrate into maltose, there was no increase in the number of non-dialysable fragments present. This supports the theory of end-wise fission of maltose groups from the starch chain-molecule as also does the hydrolysis of a dextrinic acid, from malto-dextrin, by malt diastase to a dextrinic acid of shorter chain-length.[43] Myrbäck[44] found that starch whose reducing groups had been converted into —COOH groups by prolonged action of iodine and caustic soda was split by α- and β-amylases in the same manner as the original starch. Örtenblad and Myrbäck[45] confirmed this with wheat starch dextrin and takadiastase, and from the above observations it would appear that the aldehydic end of the chain remains intact throughout the fission process.

The first glucosidic bond in the chain-molecule of starch cannot be severed by β-amylase since glucose is never found. As maltose is the sole reducing product formed it follows that the fission must occur between the second and third glucose units in the chain. One can assume with Hanes[46] that the enzyme contains two active groups of which one attaches itself to some atomic grouping in the first glucose unit, whilst the second active group attaches itself to the second glucosidic bond. According to Hanes[46] the distance between the active groups would be the length of a maltose molecule, i.e. 10 Å.

Another explanation would be that, for some unknown reason, the second glucosidic linkage was particularly unstable or had become so by virtue of the loose combination with the enzyme. Concurrent with the fission of the maltose, reversion to the β-form must take place by Walden Inversion and there are, at present, no chemical grounds for considering the formation of β-maltose in this manner improbable.

Blom, Bak and Braae[30] consider the chain-length of the starch

molecule to be 30 glucose units, and in that case the degree of fission of 53 per cent., which is observed at *pH* 3·4, would correspond with the splitting off of 8 maltose fragments. Conversion to the extent of 60 per cent. and 66 per cent. would correspond to the splitting off of 9 and 10 maltose units, respectively.

The reason for the cessation of action at this point may be due to structural alterations brought about by the enzyme when nearing the sixteenth or eighteenth glucose unit, whereby the enzyme can produce no effect (Hanes). K. Myrbäck[48] suggested that both the fatty acids and the phosphorus in starch might stop the enzyme action and Samec[49] concluded that the phosphorus in potato starch blocks the action of the enzyme, since Schoch[50] has shown that the fatty acids are not chemically linked to the starch and therefore unlikely to exert an inhibiting action. Even if the fatty acids were linked Taylor and Sherman[51] concluded that lipase-free amylase could still attack the linkages between the fatty acids and the starch molecule. With regard to the inhibition by phosphorus, Pringsheim and Ginsberg[52] have reported that they obtained complete hydrolysis of starch without liberating any free phosphoric acid. V. D. Martin, N. M. Naylor and R. M. Hixon[47] have also concluded that neither fatty acids nor the phosphorus appear to be the groups inhibiting the action of β-amylase at 60-70 per cent. conversion of starch to maltose. O. E. Stamberg[62] thinks the phosphorus may retard β-amylase action but not completely block it, whilst K. H. Meyer, Bernfeld and Press[63] think that action stops when the point of attachment of side chains is reached. This appears to be the most tenable suggestion so far put forward and with amylopectin further β-amylase action is stopped at the branch points so that limit dextrins are obtained which are indeed resistant to further attack by β-amylase. Another explanation is that aggregation of the macromolecules in ' bundles ' takes place, such that the ' bundles ' consist not of whole molecules but of their enzyme-resisting portion, the ' straggling ' or ' over-hanging ' portions of the molecule being readily attacked by the enzyme. This theory is somewhat similar to that suggested by G. V. Caesar on chemical grounds (see Chapter 12).

A further factor in favour of the latter explanation is the restoration of ' splittability ' on autoclaving. The last 12 glucose units at the aldehydic end of the molecule take part in the formation of these aggregates. An investigation of the chain-length of amyloamylose will be of interest in that it would show if it was composed of macromolecules containing 30 glucose units, which appears to be the case with all other fractions and undegraded starch. If this is found to be the case it will be clear

that the macromolecules can exist in a form which is almost free of the structure which causes the action of β-amylase to cease when about 60 per cent. conversion has been reached. Hanes suggests that the amylases which bring about liquefaction first split the bonds between the macromolecules and also that phosphoric acid is concurrently liberated, the β-amylase then shortening the chain until this process is halted for some unknown reason at 53-60 per cent. conversion. In view of the relative simplicity of the action of β-amylase on starch further study of this action appears to offer the best prospects of elucidating the mechanism of amylolytic action.

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Chapter 21

THE ACTION OF α-AMYLASES ON STARCH

W. Syniewski[1] and others (*vide supra*) showed that malt extract contained an enzyme which was unaffected by moderate heating whereas the saccharifying enzyme was heat labile. This enzyme is now known as α-malt amylase and it readily destroys the iodine coloration capacity of starch. This amylase belongs to a class of dextrinogenic amylases which are characterised by the negative mutarotation of the products of fission. Into this class the amylases obtained from the pancreas, saliva, mould enzymes of *Aspergillus oryzae* and amylases from the livers of various animals also fall. Whilst some workers have considered that these amylases are practically pure dextrinogenases, Giesberger[2] obtained a violet ring besides a broad colourless diffusion band, using Wijsman's diffusion method with the first three types, which would seem to indicate the presence of small amounts of β-amylase. The presence of maltase may also occur (*vide infra*). This might well be the cause of the widely different conclusions reached by different workers on the subject of the beginning of 'inhibition'. On the other hand, as far as mutarotation is concerned, no considerable disturbance appears to be occasioned. In general α-amylases possess the capacity of liquefying starch pastes, during which reaction there is no change in the colour of the iodine-reaction nor is there any appreciable increase in the reducing properties of the media.

Kuhn found, with pancreatic amylase, that on stopping the action when it had reached 53 per cent. conversion the reducing power remained constant but the rotation became definitely less. From hypoiodometric estimation of the maltose present and from the optical rotation that should be shown by this amount of maltose, it was found that a solution of similar strength, containing α-maltose, should have a higher rotation, but that the calculated total loss in rotation was equal to the difference in rotation between α-maltose and α-β-maltose in the given concentration.

O'Sullivan, in 1876, using diastase, i.e. α- + β-amylase, found that pre-heating to 70-80° C. reduces the amount of maltose formed very appreciably, and that under certain conditions

product consists almost entirely of reducing, non-crystallisable dextrins. This is of importance in the adhesive industry (*cf.* Vol. II).

It is now realised that in many of these early experiments the workers were dealing with preparations which varied in composition from isolated α-malt amylase to mixtures rich in β-amylase. The dextrins produced in these cases underwent further hydrolysis when acted upon by unheated malt extract.

The dextrins produced vary in their complexity and their solubility in hot or cold aqueous alcohol of varying composition, the latter property being closely connected with the former. Amylo- and erythrodextrins show low solubility in aqueous alcohol, low reducing power but high optical rotation and molecular weight, whereas the more highly degraded products, the achro- and maltodextrins, show increased solubility and reducing power and lower rotation and molecular weight. All the products, amylo-, erythro-, achro- and maltodextrins, are hygroscopic and are fermented only slowly or not at all by yeast. The iodine reaction of these products varies from purple through red, brown to colourless progressively with decrease in the chain length or molecular complexity of the dextrins. The point at which no iodine reaction is obtained is known as the achroic or achromic point.

Amylo- or erythrodextrins appear to constitute the main product in the action of α-malt amylase on starch up to the achroic point (Syniewski[14]). The achro- and maltodextrins appear to be formed only slowly or when β-amylase is present. Pollak and Tychowski[3] obtained an 87 per cent. yield of the amylo-type of dextrin when acting on amylose with pre-heated malt extract. Hanes[4] considers that the first type of dextrin is invariably formed by the action of α-malt amylase on starch, and that it consists of fragments of the starch chain of about 6 glucose units in length and the second type to have a chain-length of some 4 glucose units. The hexaose of Waldschmidt-Leitz and Reichel[5] formed by the action of pancreatic amylase on rice starch and on erythroamylose has properties in close agreement with those demanded by a 6-unit dextrin.

Pigman has estimated the fermentable matter formed by amylose action by fermenting it with yeast and determining the alcohol produced (see p. 278). He finds that preparations from barley and kaffir malt carry the conversion of starches to 87-91 per cent. of the theoretical quantity of sugar. This figure is appreciably higher than the values obtained by other workers who ed reducing sugar determinations to estimate the sugar formed.

This worker also finds that there is no appreciable increase in the degree of conversion for preparations which are known to contain large amounts of β-amylase, e.g. barley malt. He considers that the difference between the results obtained by the fermentable sugar and reducing sugar determinations may be ascribed to a lack of quantitative significance of the reducing sugar method when applied indiscriminately to mixtures of maltose, dextrins and dextrose such as are produced by the action of α-amylases. Recently Hopkins and Kulka[50] have obtained a conversion of starches to reducing sugar of about 90 per cent. with malt α-amylase.

When dealing with the α-amylases optical rotation more readily shows differences than reducing power, but the differences disappear after soda has been added to the solution. After a certain period of time no further mutarotation takes place. Using electrodialysed starch solutions there is a sharp inflection point at the beginning of the curve of optical rotation against time of action of pancreatic amylase. Kuhn, Ohlsson and Swaetichin[6, 31] also found that the optical rotation values before and after the addition of soda to the reaction mixture to lie close together when takadiastase is used and conclude that it should be classed as an α-amylase in spite of the presence of maltase. The dextrinogenic amylase of E. Ohlsson also gives negative mutarotating hydrolysis products but the absolute values of rotation are much lower than those obtained by Kuhn with pancreatic amylase. There is, as yet, no conclusive evidence that enzymes of the α-malt amylase type exist in isolation in any tissue.

J. Blom, A. Bak and B. Braae[7] consider the bacterial preparation, Superclastase, which has a starch liquefying action, belongs to the α-amylase type, and Jancke and Holota suggest the same of Biolase.[15] H. Lüers and A. Lother[8] have shown that an energetic liquefying action is exhibited by this enzyme unaccompanied by saccharification. Freeman and Hopkins,[9] working with soluble starch and pancreatin, filtered the reaction mixtures through an iron phosphate gel which removed not only the enzyme and the turbidity produced during the hydrolysis (*vide infra*) but also the dextrins of large molecular size, so that optical measurements on the products of fission were simplified. These workers observed a falling mutarotation during the hydrolysis of amylo- and erythro-amyloses, glycogen and α-amylodextrin with α-malt amylase, and according to this the type of mutarotation is characteristic, not of the substrate, but of the enzyme employed.

The course of hydrolysis brought about by α-amylases has

been determined by these workers[10] and by Klinkenberg,[11] Holmberg[12] and Hanes[13] by measurement of the reducing values. The action of this group of enzymes is characterised by two distinct phases, a rapid initial reaction which is checked when an R_M value of 30-40 per cent. is reached and a subsequent slow reaction in which the reducing power of the reaction mixture slowly rises. Both the initial velocity and the final degree of hydrolysis are appreciably increased when the concentration of enzyme is increased. This suggests that the enzyme may be progressively deactivated, such deactivation would partially explain the checking of the initial, rapid hydrolysis and the dependence of the final limit of hydrolysis upon the initial concentration of enzyme used.

This inhibition occurs whichever type of α-amylase is used, but the greater the amount of erythro-substances present the lower is the degree of saccharification produced. Table XXXVI shows the results obtained by various workers, using different substrates and types of α-amylase.

S. J. Pronin[19, 20] obtained a degree of hydrolysis greater than that obtained by Freeman and Hopkins, but he used relatively large quantities of enzyme, so that in view of the possible contamination by β-amylase his results are not completely conclusive. It will be noted that the limit of hydrolysis in these cases fall close to those obtained by the use of diastase or mixtures of α- and β-amylases.

Hanes[21] considers that with pure α-malt amylase, prepared by the method of Holmberg or from certain malts by Ohlsson's method, the initial rapid phase would cease at 28-30 per cent. apparent conversion and that the hydrolysis limit would not exceed an R_M value of 50 per cent.

Although salivary and pancreatic amylases appear to induce the initial cleavage of starch into reducing dextrins, with the concomitant disappearance of the iodine coloration, the degradation appears to continue beyond the point at which the reaction is checked in the case of α-malt amylase. This important difference appears to indicate a higher affinity, on the part of animal amylases, for the less complex products of degradation in relation to their affinities for the more complex starting materials, than is the case with α-malt amylase. One point which seems to be in favour of this attractive but incompletely supported hypothesis is that the *achroic* R_M *values* found by C. Hanes and M. Cattle[22, 23] for α-malt amylase is 29-32 per cent. and is independent of the substrate concentration. The *achroic* R_M *values*, determined spectrophotometrically for *Aspergillus*, pancreatic

TABLE XXXVI

Hydrolysis of Starch and Derivatives by α-Amylases

Substrate	*α-Amylase Used*	*Limits of Hydrolysis*	*Observer*
Potato starch	Bacterial	48	J. Blom, A. Bak and B. Braae[25]
Lintner soluble starch	Malt	75-78·5	van Klinkenberg[11]
,, ,, ,,	,,	60	C. S. Hanes[13]
,, ,, ,,	,,	77-84	G. G. Freeman and Hopkins[10]
,, ,, ,,	,,	86·9	M. Samec[16]
,, ,, ,,	Salivary	75·83	H. J. Vonk and I. P. Braak[17]
,, ,, ,,	Pancreatic	54	,, ,,
Amylo-amylose	Malt (Klinkenberg)	93	M. Samec[16]
,, ,,	Pancreatic	101	M. Samec and E. Waldschmidt-Leitz[18]
Erythro-amylose	Purified pancreatic	70	,, ,,
,, ,,	,, ,,	70	E. Waldschmidt-Leitz and Reichel[5]
,, ,,	Malt (Klinkenberg)	80	M. Samec[16]
Lintner soluble starch	High concentration of pancreatic	86-88	S. J. Pronin[19, 20]

and salivary amylases increase with increasing substrate concentration.

Pigman[51] from his work concludes that there may be a fundamental difference in action between the various liquefying amylases. Cereal α-amylases and the *aspergillus* enzymes are able to break down starch completely to fermentable material in the presence of yeasts but pancreatic and *B. meserenticus* enzymes, although showing marked liquefying ability are unable to bring about complete amylolysis to fermentable substances in the presence of yeast. *B. macerans* amylase liquefies starch with the production of Schardinger dextrins which are cyclic hepta- and hexasaccharides as attested by many workers.[52–56] Whether these products are formed during the first reaction or are the result of resynthesis of the products of amylolysis has not yet been determined. Pigman therefore proposes classifying the α-amylases into two main groups (*a*) cereal and *aspergillus* α-amylases and (*b*) pancreatic and *B. mesentericus* α-amylases to which may possibly be added salivary and *B. macerans* amylases.

An outstanding feature of α-amylase action is the destruction of the iodine coloration of the solution, which undergoes a rapid shift through blue, violet, reddish-brown, orange to yellow. The last two colour changes take place slowly making determin-

tion of the achroic point difficult. R. Kuhn and M. Samec have given the effect of α-amylase action on various starch substrates and their results are given in Table 17. It will be seen that, despite the different substrates used, the *erythro-state* is reached after about a value of R_M = 30 per cent. has been attained and the achroic state after a value of R_M = 55 per cent., while the course of the hydrolysis, using substrates which have different initial iodine colorations, is also clearly brought out in the table.

With β-amylase the increase in reducing power runs reasonably parallel with the disappearance of the colour given with iodine, but with α-malt amylase these curves diverge considerably. The work of Hanes and Cattle[22, 23] is of great interest. By measuring the extinction coefficients of the reaction mixture after the addition of iodine solution they showed that for starch and a number of dextrins the extinction coefficients were proportional to the concentration of the starch products present. With α-amylases a decrease in the extinction of the longer wavelengths occurs in the initial stages of the reaction, but later the value decreases throughout the whole spectral range, the decrease at the red end of the spectrum still being predominant, so that the absorption peak drifts towards the blue end of the spectrum. This is in sharp contrast to the extinction curve obtained for β-amylase, where a decrease over the whole spectral range is observed, but the peak of maximum absorption and the general form of the curves do not vary even up to R_M values of 59 per cent.

It should be noted that with α-malt amylase the achroic point is reached in that range of hydrolysis in which the time reducing-value curve shows a sharp inflection point showing the beginning of the 'zone of inhibition'. At the achroic point dextrins containing 7 or 8 or more glucose units are probably absent, and thus the ideal dextrinogenic process would result in the transformation of the whole substrate into dextrins of 6-7 glucose units, with the liberation of the minimum number of reducing groups. In this case the lowest attainable achroic R_M value would, assuming the full reactivity of the aldehydic groups, be 29·7-34·6 per cent. The values given by different workers for the inhibition zone mentioned above are about 34 per cent.,[24] 34-38 per cent.,[13] 33-40 per cent.[25] and 36 per cent.[3, 11] T. G. Halsall, E. L. Hirst, L. Hough and J. K. N. Jones[58] found that the limit dextrins produced by pure crystalline β-amylase have much higher molecular weights than those produced by crude enzyme preparations.

The Intermediate Products of α-Amylase Action.—M.

TABLE XXXVII

*Pancreatic Amylase**				*Pancreatic † Amylase*		*α-Amylase †*						
Amyloses				*Amyloses*		*Amylo-amylose*			*Erythro-amylose*		*Amylodextrin*	
Time (min.)	*I coloration*	Rm.	K ·10⁴	*I coloration*	Rm.	*Time (min.)*	*I coloration*	Rm.	*I coloration*	Rm.	*I coloration*	Rm.
7·5	Blue	7·5	45·3			0	Blue	1·99	Violet-red	9·0	Orange	0·98
21	Blue	16	36·2						Bluish			
				Reddish-blue	25		Greenish					
40	Reddish-blue	28	35·8	Deep red	32							
63	Reddish-brown	40·5	25·9	Reddish-brown	39	60	Yellow	41·4	Reddish-orange	17·1	Orange	11·5
						120	Yellow	48·1	Reddish-orange	18·6	Orange	15·9
				Brown	45							
						180	Yellow	52·0	Orange	25·0	Orange	16·9
290	Colourless	56·5	12·5	Yellow-brown	49							
						300	Yellow	53·8	Orange	33·1	Orange	17·5
1800	Colourless	70	2·9	Colourless	54	420	Yellow	58·8	Orange	35·4	Orange	17·5
3300	Colourless	74·5	1·8									
∞	—											

* R. Kuhn's results. † M. Samec's results.

Samec and M. Blinc[26] are not in full agreement with C. Hanes.[27] They[16] obtained an even lower achroic R_M value, e.g. 16·4 per cent., and consider that achrodextrins having appreciably higher molecular weights than correspond with molecules of 6-7 glucose units exist (Biltz,[28] Samec[29]).

During the first rapid stage of the degradation of starch with α-amylase reducing dextrins, supplying the bulk of the reducing value of the mixture, are formed. It must be emphasised, in view of the frequent assumption to the contrary by a number of workers, that maltose is neither the sole, nor the principal, reducing substance formed in all stages of the hydrolysis.[57] The quantitative investigation of the intermediate products has been undertaken by Ohlsson.[30, 31] By dialysing the reaction mixture when the iodine coloration was violet and the R_M value was 23 per cent. he was able to establish, by osmotic pressure measurements, that the number of slow or non-dialysable particles had greatly increased and that these relatively complex fragments contribute the bulk of the reducing power of the solution.

Freeman and Hopkins,[32] by fractional precipitation of two digests with a violet iodine coloration and R_M values of 20·6 and 25·6, respectively, gave 80 per cent. of dextrins having R_M values of 20·3 and 23 per cent. respectively, which are very close to the initial R_M values of the solutions. The remainder of the material consisted chiefly of products more complex than maltose, but a little of the latter was probably also present. The dextrins obtained by the various workers have been described above.

M. Samec and R. Rakusa[33] consider the fragments formed in the initial stage of α-malt amylase hydrolysis are nearly uniform. Under suitable conditions a very rapid drop in viscosity, with scarcely any other change in the properties of the starch solution, is to be noted. Myrbäck,[34] using takadiastase on corn starch, claims to have isolated from the reaction mixture dextrins which were trisaccharides and discusses the importance of this in connection with the structure of starch.

Ross and Romijn[35] found that the rate of fission of 1 per cent. potato, rice, wheat and soluble starch solutions decreased in that order when acted upon by pig-pancreas amylase, but using dog-pancreas amylase the order of the last two starches was reversed. In each case, however, the maximum R_M value was 50 per cent. The first amylase was the quicker acting on potato starch.

G. E. Glock[36] used pancreatic, malt and salivary α-amylases on wheat, rice, maize and potato starches and glycogen. The [illegible] very large quantities of enzyme showed maltase action.

When used in the usual concentrations this was negligible but with liver amylases of various animals, including humans, maltase action was never entirely absent. Thorough washing of rat liver with saline solution before preparing the amylase gave a preparation having no maltase action, and it may be that in the case of liver amylases the presence of blood is responsible for the maltase action reported. In the initial stages the action of ptyalin and pancreatic amylases appears very similar to that of α-malt amylase in that reducing dextrins are produced and the iodine coloration is destroyed. Whether the initial fragmentation is similar or not is obscure, but irrespective of the initial size of the primary fission products these are further degraded very rapidly by the two animal amylases. The end of the primary or dextrinogenic stage is therefore not marked by any slackening or inhibition in the rate of hydrolysis such as is shown by α-malt amylase.

The velocity with which the individual changes take place varies with the starch as follows, the starches being placed in order of decreasing rate of change :—

Achroic point	Glycogen ; potato, maize, wheat and rice starches.
Amount of—	
substrate hydrolysed	Potato, maize, wheat and rice starches ; glycogen.
reducing dextrins and maltose formed .	Wheat, potato, maize and rice starches ; glycogen.
maltose formed	Wheat, rice, potato and maize starches ; glycogen.

Samec has suggested that as the amounts of maltose and dextrin vary from starch to starch that a constant value of the quotient—*starch hydrolysed/maltose formed*—would show that maltose is formed successively, but if it fell in value during the course of hydrolysis it would show that the dextrins were more or less completely converted into maltose.

According to Blom, Bak and Braae,[37] during the action of bacterial α-amylase only maltose is formed at the beginning of the hydrolysis, and a maximum of 23 per cent. is reached when 40 per cent. starch fission has been effected. From this point onwards they consider that the number of molecules of reducing dextrin do not increase but that maltose is split off from these dextrins. Kitano,[38, 48] using crude takadiastase, claims to have obtained a trisaccharide which gives maltose and glucose on hydrolysis and, according to this worker, the achroic point is very rapidly reached with pure takadiastase and the final product is maltose which is found quantitatively.[39]

Takadiastase has been found by H. Weinmann to only partly hydrolyse commercial dextrins, prepared by acid hydrolysis, although under the same conditions starch and maltose were completely hydrolysed to glucose. The greater the degree of conversion of the dextrin, the greater the resistance to hydrolysis. Rice starch dextrin, formed by the action of ptyalin from saliva was almost completely hydrolysed.

When using α-malt amylase or salivary α-amylase with a starch substrate which has not been strongly peptised a small amount of flocculent material is produced just before the achroic point is reached. If the solution is boiled and cooled the iodine coloration is found to have returned, and on the addition of fresh amylase the hydrolysis continues. V. D. Martin[40] noted that the material which flocculates and the precipitate formed by the addition of 60 per cent. alcohol to the filtered solution have the same reducing values. Incidentally, Martin and co-workers[41] conclude that neither the fat nor the phosphorus content appears to be responsible for the cessation of action of β-amylase at R_M 60-70 per cent. fission. Broeze[42] considers the flocculent precipitate to be formed owing to association of molecules whereby the iodine reacting groups in the molecules are masked. As it occurs with starch which has not been thoroughly peptised we may conclude that the aggregates are already present in the solution and may possibly be protected by the fully peptised material (amylose). When the amylose has been destroyed by amylase action the iodine reaction is destroyed and the aggregated material flocculates and forms a ' haze ' or a precipitate. Strong heating of the solution in an autoclave brings about further disaggregation, freeing more iodine-reacting groups, thus restoring the iodine coloration and further material is made available for amylolysis.

The Action of α-Amylase on Unpasted Starch.—As previously mentioned the unpasted starches all appear to be ' immune ' against amylase attack except when the granules are injured by grinding. Pozerski[43] has shown that in this case it is the solubilised portion which is attacked and not the suspended fragments. Ross and Romijn[35] found that amylase from dog or pig pancreas had no action on granules of potato, wheat or soluble starch.

Weichsel.[44] however, claims that even unpasted starch granules are attacked by takadiastase at 37° C. and pH 4·9, wheat starch being liquefied in 24 hours, canna starch saccharified to an extent of 90 per cent. in 5 days but potato starch remained unaffected even after several weeks. If treated with a solution of a non-

poisonous swelling agent at 37° C. for a few days he found diastatic action could then take place.) The same effect was obtained by pre-treatment with alcohol for 24 hours or acetone for 3 days. The effect of these liquids is to cause the appearance of fissures in the granules very similar to those observed by the author with intensely dried starch, so that the enzyme can enter the granule. On the other hand, intense drying of potato starch over phosphorus pentoxide for several months was found by Weichsel to render the outer membrane swellable in cold water and thus initiate amylolysis from without the granule.

W. Ziese[45, 46] finds that pancreatic, and to a lesser extent salivary amylase will attack oxyethyl starch giving an appreciable fall in viscosity but little change in the optical rotation or reducing power. The products of the reaction do not diffuse through a cellophane membrane. This worker considers that dehydration takes place as an intermediate step in this reaction. It is of interest to note that takadiastase is practically without effect on oxyethyl starch.

The Mode of Action of α-Malt Amylase.—The action of α-malt amylase on starch is a more complicated process than the action of the β-amylase. During the first stage the bulk of the substrate is converted into complex reducing dextrins and the iodine coloration shows a progressive alteration, the achroic point being reached when the reducing power is about 29-32 per cent. of the theoretical maltose. The chemical and physical constants of the dextrin fraction having no iodine coloration suggest a *mean* chain-length of 6 glucose units. It would appear that α-malt amylase can break up the long chains present into fragments having a chain-length of 6 glucose units, but whether this fission takes place in an endwise manner as in the case of β-amylase action is not deducible at present.

After the primary fission to dextrins a slow hydrolysis of the dextrins appears to take place until the reducing power is of the order of 50 per cent. of the theoretical maltose value. If the substrate for this second slow hydrolysis is assumed to be a 6-unit dextrin, produced in the first stage of the reaction, then the final reducing power indicates that only about one more linkage in each dextrin molecule is capable of being hydrolysed by the amylase. Neither the course of this secondary reaction nor the composition of the products obtained can, at present, be precisely defined. Whether or not the slow rate of the secondary reaction is due to the enzyme having only a low affinity for the reducing dextrins or to the slow breakdown of the enzyme substrate complex are other questions at present unanswerable.

The rapidity of the first phase of the hydrolysis by α-amylase suggests that relatively long portions of the chain structure enter into the enzyme-substrate complex. As suggested by Hanes in the case of β-amylase it seems quite reasonable to suppose that there are two active groups in α-amylase by which attachment to the long-chain molecule can be accomplished, followed by fission of at least one of the points of contact. As dextrins of 6 glucose units in length form a considerable portion of the products of hydrolysis these active centres must be some distance apart, and if fission takes place at the points of attachment then the distance between them will be in the neighbourhood of 6 glucose units. Hanes points out that one objection to this hypothesis is that it assumes an unlikely degree of structural organisation.

To overcome this Hanes[47] suggests that the starch chain might conceivably be in the form of a spiral of 5 complete turns and that 6 glucose units complete one full turn. Every seventh linkage, i.e. the linkages which are split, would then fall in close *lateral* proximity although separated along the chain by 6 glucose units. These two points being fairly close together the α-malt amylase can readily attach itself by means of its active groups. Cleavage at these two points would liberate one complete turn of the spiral containing 6 glucose units. As dextrins with 6 to 7 glucose units give no iodine coloration this conception of a spiral molecule leads to the suggestion that the iodine coloration depends on the presence of more than one complete coil of the spiral. G. V. Caesar[49] and M. L. Cushing also have concluded that amylose has a helical-spring configuration which is well brought out in the Fischer-Hirschfelder atomic models they prepared.

More work on the configuration of the starch molecule and a rigid characterisation of the products of the first stage of α-amylase action on starch is required before anything final can be decided on the mode of action of amylases on starch. The hypothesis of Hanes certainly shows that it is unnecessary to postulate different linkages to explain away the peculiarities of enzyme action.

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Chapter 22

THE ACTION OF MIXTURES OF α- AND β-AMYLASES ON STARCH

Malt extract probably exemplifies the best-known natural mixture of α- and β-amylases. The relative proportions and influence of each type of amylase varies according to the source and method of preparation of the malt extract. Sherman and Schlesinger[1] have shown that the disappearance of iodine reaction cannot be used as a criterion of starch splitting capacity of malt extracts, as the action of the α-amylase is affected to a different degree to that of the β-amylase by different factors.[2] E. Ohlsson showed the ratio of α- to β-amylase to vary in different malt extracts and this has been confirmed by Fricke and Kaja.[3] That conflicting statements have appeared on the action and the higher limits of degradation of starch when acted upon by malt extracts is not surprising in view of the heterogeneous nature of these extracts.[4, 5] R. H. Hopkins and co-workers[21] point out that not only can the proportions of α- and β-amylase vary in a given sample of malt but also that the extent to which the latter hydrolyses starch depends upon the severity of the pre-treatment given to it during its preparation. These findings agree very well with the suggestions of Lampitt.

H. Kühl[6] has observed a greater formation of erythrodextrin from rye starch than from wheat starch when these were acted upon by strong malt extracts. The achrodextrin stage was, however, reached in both cases. A. Tychowski[7] found that malt extracts prepared from oat, rye and wheat hydrolysed solutions, prepared by autoclaving, of various types of starches to the same extent. Increasing the temperature from 20° to 55° C. and increasing the enzyme concentration favourably influenced the hydrolysis but the final amount of maltose produced was unaffected.

Sherman and Baker[8] found potato amylose to be practically completely saccharified by malt extract, but amylopectin shows greater resistance and only a hydrolysis limit of 63 per cent. is finally attained. Lintner starch and soluble starch prepared by autoclaving lies between the two. H. Pringsheim and A. Beise[9] confirmed these observations, using malt amylase in the presence

of 'amylase-complement'. They found amylose to be completely saccharified, but the hydrolysis of amylodextrins could only be brought to completion in the presence of the 'amylase-complement'. Pigman's work in this direction has already been mentioned on p. 278.

H. Lüers and W. Wasmund[10] found that small changes in the starch substrate cause a notable alteration in the reaction-constant. Samec and Blinc[11] and E. Waldschmidt-Leitz[12] have obtained similar results with amylo- and erythroamyloses when using malt extracts.

Freeman and Hopkins[13] investigated the action of artificial mixtures of α- and β-amylases on glycogen, soluble starch and amylodextrin. In small concentrations the effect of the amylases in the mixtures appeared additive, but in higher concentrations, and especially where the proportion of β-amylase is increased, the degree of hydrolysis in the first stage is greater than the sum of the actions of the two components separately. This is probably due to the fact that β-amylase can act readily on the residue from the total action when this residue has been acted upon by the α-amylase. The hydrolysis limits, using a mixture of the enzymes, is some 10-12 per cent. higher than the sum of the action of the two components separately, and Freeman and Hopkins never obtained a 100 per cent. hydrolysis. In view of the explanation of amylase action advanced by Hanes this is what might have been expected. A. Joszt and A. Kleindienst[14] studied the first rapid stage of saccharification and found some 30 samples of distillery malts to attain a degree of hydrolysis corresponding to 51·7 to 56·2 per cent. of the theoretical maltose groups in this stage.

Pigman has noted that less fermentable matter is produced by the joint action of two amylase preparations than occurred when either was used alone. He attributes this to the synthesis of unfermentable material from the products of diastatic hydrolysis. Treatment of dextrose and of maltose solutions with various enzyme preparations showed that none of the enzyme solutions synthesised unfermentable matter. With maltose, however, enzymes from fungal (*Aspergillus*), bacterial (*B. mesentericus*) and pancreatic preparations produced considerable unfermentable matter. In the presence of yeasts, however, the material produced by the fungal enzymes is reconverted into fermentable matter, as shown by the amount of alcohol produced, whereas in the cases of the bacterial and pancreatic enzymes only a portion is thus reconverted. The fungal, pancreatic and bacterial preparations used by this worker were mixtures of enzymes so that

the synthesising action cannot be ascribed only to the amylases present. Stark[22] found that pancreatic and salivary amylases acting on starch showed the presence of more unfermentable dextrins in the later stages than in the intermediate stages, a continual shift in the nature of the hydrolysis products being evidenced. This may be due to the synthesising action of the amylase preparations.

Tarr and Hibbert[23] report numerous examples of the production of dextrins and levans when acting on sugar solutions with enzyme solutions prepared from moulds or bacteria but enzymatic constituents present in minor amounts, and not amylases, may well be the operative agents here.

Blom, A. Bak and B. Braae obtained a hydrolysis limit of 80 per cent., using a mixture of bacterial α- and β-barley malt amylase on potato starch. If a high proportion of an amylase-complement is present it is possible to obtain complete hydrolysis of the starch to a dissaccharide (Samec and Blinc).[11]

H. Pringsheim and J. Leibowitz[15] considered that the action of a combination of α- and β-amylases on starch should produce glucose, but P. Rona and J. Hefter[16] and Samec[12] dispute this. Samec worked with pancreatic and malt amylase mixtures on erythro- and amyloamyloses as substrates and obtained a hydrolysis limit of 85 per cent. with erythroamyloses and 101 per cent. with amyloamyloses. Ordinary starch is hydrolysed until the reducing value showing a limit of 80 per cent. has been reached

The results of following the reaction by *the iodine coloration* are interesting. The fading of the blue colour, as well as the red, is more rapid the richer the substance is in amylose, and at the achroic point the reducing value is higher the more amylose there was present at the start. If this is decreased in favour of amylopectin the reducing value at the achroic point falls to an extent dependent on the reduction in the percentage of amylose present at the start.[8]

H. von Euler and O. Svanberg[17] obtained a 71 per cent. saccharification at the achroic point, using their dialysed malt extract. According to Effront[18] no reducing dextrin is produced in the first stage of the malt hydrolysis but appears in the second and longer stage. The action of mixtures of α- and β-amylases on starch has also been studied by H. and W. Brintzinger[19] and by R. Weidenhagen and A. Wolf.[20]

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Chapter 23

THE KINETICS OF AMYLASE ACTION

As amylases are catalysts and thus alter the rate of reaction it is necessary to study their influence on the velocity of reactions and the influence of various factors on their action. Any process which tends to reach an equilibrium becomes slower and slower the nearer the point of equilibrium. It might be thought that the rate of hydrolysis of starch was proportional to the number of starch molecules present and that the reaction should therefore be unimolecular. It must be kept in mind, however, that when the reaction is activated by amylase the process occurs in a heterogeneous system involving reactions at the surface of the amylase and of the starch micelle. If, then, we find a particular amylase action apparently obeying the unimolecular law it cannot be ascribed to the attainment of a homogeneous system but must be due to some other cause. The kinetics of amylase action has been discussed by several workers.[20, 45–47]

Malt Amylase

(*a*) **Saccharogenic Action.**—1. *Measuring the Reaction Constant.*—The saccharification of starch is a progressive reaction in which fission takes place, producing maltose and residues which are then subjected to further hydrolysis. It is immaterial at this point to consider whether maltose units are progressively split from the end of a long chain or whether the long chain is broken at random along its length into pieces which, in turn, undergo further fission.

Kjeldahl stated[1] that the relative diastatic power of two solutions is expressed by the copper reducing power produced in a given time when the enzyme solutions act on the same weight of starch at the same temperature, provided always that the reducing power is not allowed to go beyond R_M 40-49. An examination of Kjeldahl's data shows that the reaction is not strictly linear as far as the reducing value of R_M 40 but is sufficiently so for all practical purposes for measuring commercial diastase preparations. It is, however, very nearly rectilinear up to the reducing value of R_M 29.

C. J. Lintner and F. Eckardt[2] confirmed Kjeldahl's results for soluble starch. A. Janke and J. Holota,[3] however, consider that the linear course does not appear and that the reaction is unimolecular up to reducing values of R_M 30 at 30° C. and R_M 45 at 50° C. Beyond these limits, or for total hydrolysis by any diastatic preparation, the reaction constants diminish and negative auto-catalysis prevails.

H. T. Brown and T. A. Glendinning[4] and H. Lüers and W. Wasmund,[11] using soluble starch, found that at the beginning of the reaction there was a marked increase in the constant with the progress of hydrolysis, and after a value of R_M 30-40 had been reached the reaction followed a unimolecular course. In their early experiments Brown and Glendinning were convinced that the rate of change did not conform to the logarithmic formula for unimolecular action, but that there was a progressive increase in the value of the 'velocity coefficient' which, at first sight, appears to differentiate the mode of action of diastase from that of invertase which gives the logarithmic form characteristic of a unimolecular reaction. Following the work of A. Brown and V. Henry they re-examined their results and concluded that one fundamental law can express the rate of change in all enzyme reactions which can be studied quantitatively with sufficient accuracy. They used both change in optical rotation and cupric reducing power to follow the reaction, but owing to the comparatively small change in the former the method is the less accurate of the two.

They calculated the coefficient of velocity directly or else the results were plotted as a *time proportion of unhydrolysed substrate* curve. If the reducing power of totally hydrolysed starch solution of unit volume is regarded as unity and x is the ratio of the reduction observed at any given time t from the start of the reaction, then if the time curve representing the course of the hydrolysis is logarithmic then $\frac{1}{t} \log \frac{1}{(1-x)} = k$, where k is the constant representing the coefficient of velocity of hydrolysis and t is the time elapsing from starting the reaction.

Throughout the reaction there is a steady increase in the value of k showing that the course of hydrolysis does not follow the logarithmic expression. If we assume that the normal curve for the rate of change is logarithmic but that secondary disturbing causes have caused some modification of the form, then the secondary conditions must be such as to produce a constant

acceleration of the coefficient of velocity of change, i.e. in any given time interval there is more of the residual substrate hydrolysed than there should be according to the logarithmic formula. Brown and Glendinning thought at first that the intermediate products were probably more easily hydrolysed than the more starch-like products, thus giving a constant increase, but were able to demonstrate experimentally that this was not so.

The time-curve expression of the rate of hydrolysis is approximately a straight line from the initial value up to values of R_M 30-40. In this portion of the curve the amount of hydrolysis is approximately proportional to the time of reaction, the amount transformed being a linear function of the time. The curve then gradually changes over into a logarithmic form. C. Philoche[5] considers the unimolecular 'constant' decreases up to R_M 30 and then becomes constant and V. Henry[6] considers that the value of k varies about a mean value.

H. C. Sherman and J. A. Walker[7] considered the reaction to follow a unimolecular course in the early but not in the later stages, but they used very low enzyme concentrations and recent work has shown that it needs especially limited requirements of substrate and enzyme concentration in order to reach a favourable approximation to the unimolecular course (see H. van Laer[8]).

H. von Euler and O. Svanberg[9] obtained a practically constant value of the velocity constant in the initial stages by the very careful choice of working limits. They found the constant of the secondary to be about 1/1,000th that of the primary saccharification and the slowest speed of reaction was reached near the value of R_M 75. Ordinary potassium and sodium salts and the phosphate-buffer had no influence on the reaction constant.

H. von Euler and K. Josephson[10] compared the hydrolysis with malt of Lintner starch and glycogen, and from the reaction constants concluded that glycogen belongs to the erythro-class of compounds.

2. *Reaction Constant and Enzyme Concentration.*—Ch. Philoche found no linear relationship between the amount of sugar formed in a given time and the enzyme concentration. Sherman and Walker concluded from their work that within certain limits of enzyme concentration and up to values of $R_M = 50$ the maltose formation was directly proportional to the enzyme concentration. Other workers,[9, 11] have found a corresponding proportionality between the enzyme concentration and the reaction constant of the unimolecular reaction and consider that the value of *k/amylase concentration* is significant. For concentrations of amylase up to 0·1 per cent. a better constant is obtained if one considers the

value—*initial value of k/amylase concentration.* A similar consideration of higher amylase concentrations (0·1667 per cent.) shows that the production of maltose increases at a slower rate than the increase in the proportion of the enzyme used. The saccharification rate may be increased by raising the amount of amylase but the final amount of maltose formed is fairly constant (see K. Sjöberg[12]).

K. H. Meyer and J. Press[42] have studied the influence of starch and enzyme concentrations on the degradation of Zulkowski soluble starch by β-amylase. They find that the reaction velocity is independent of the starch concentration at high starch concentrations and up to a degree of hydrolysis of 30-40 per cent. at lower concentrations. For a constant starch concentration the quotient *k/amylase concentration* is constant. At high starch concentrations it tends towards a limit, the existence of which they consider indicates the formation of an addition of the enzyme with the starch which later decomposes into enzyme and starch degradation products. Application of the law of mass action leads to the conclusion that, for a given constant starch concentration, *k/amylase concentration* should be constant. When values of the quotient are plotted against log A, where A is the initial concentration of starch, an S-shaped curve is obtained. They give the dissociation constant of the starch—β-amylase compound as 0·0008 (see below). Addition of maltose was found to lower the velocity of degradation. In similar studies of the degradation of maize and potato amyloses by β-amylase the value 0·0001 was obtained for the dissociation constant of the enzyme-amylose compound. In order to obtain the same reaction velocity with amylose and with soluble starch it is necessary to use an amylose concentration 1·5 times that of starch. In the degradation of amylose by β-amylase the curve *maltose formed/time* is a straight line up to a degradation of 65 per cent. and the reaction is of zero order according to these workers.[43]

Using undegraded potato and maize starches as substrates Meyer, Preiswerk and Jeanloz[48] found potato starch to be attacked a little more easily than maize starch whilst both were more resistant than soluble starch. The velocities of degradation at 4° and 35° C. were compared and the *k/amylase concentration* found and discussed.

Hopkins and co-workers[49–52] have examined the action of β-amylase from malt and soybeans on several substrates. With potato amylose the reaction rate declined steadily with time except with some samples, considered to be partly denatured by the conditions of preparation, with which hydrolysis was incomplete.

The system followed the Michaelis and Menten formulation in certain respects. The affinity constant appears to vary with the method of preparing the amylose and the less degraded the amylose the faster the hydrolysis. They concluded that long chains are attached more readily than shorter chains. They prepared an amylose fission product[51] of mean chain length 20 glucose units which, after purification, was treated with soybean β-amylase as was the parent amylose. The Michaelis constants were found to be 5×10^{-5} and $6\text{-}8 \times 10^{-5}$, respectively, and the maximum velocities were about equal. This appears to indicate that there is little or no significant difference between the rates at which the individual long chains of normal amylose and these shorter ones are hydrolysed, both being liable to simultaneous attack and the same would appear true for the chains of varying length actually present in amylose.

Using potato amylose and amylopectin at *p*H 4-6 Hopkins and Jha[52] found β-amylase to split off maltose from amylopectin about twenty times as fast as from amylose until about 20-25 per cent. of theoretically equivalent maltose has been split off. The value of the Michaelis constant (Km) is about twice as great for amylose as for amylopectin. The hydrolysis of amylopectin is almost linear with time but that of amylose soon declines. A mixture of the two components in the ratio in which they occur naturally shows a course of reaction similar to that taken by the natural starch. Amylose diffusing from slightly swollen potato starch at 63° C. and amylose from Lintner's starch are hydrolysed at a faster rate than normal potato amylose.

3. *Concentration of Substrate.*—The concentration of substrate is more important in enzyme systems than in homogeneous systems. In the latter, according to the unimolecular reaction, the reaction constant holds over wide limits of substrate concentration, but in the former one often finds an inverse proportionality between the substrate concentration and the reaction constant, so that Kc = constant.

Henry and Ch. Philoche found that similar amounts of amylase in similar times produced more maltose from concentrated starch solutions than from dilute solutions, and H. van Laer[8] concluded that, in general, a constant of the absolute amount of maltose produced by the enzyme does not exist but, as the substrate concentration rises, a smaller increase in the amount of maltose formed occurs than might have been expected on the basis of the increased amount of substrate. C. Wirth[13] found that with increased initial concentration of starch the reaction constant decreases, and Euler and Svanberg[9] found a linear

relation throughout between the reciprocal of the substrate concentration and the reaction constant for starch solutions of 1, 2, and 4 per cent. strength, and Lüers and Wasmund have confirmed this for 0·4-6·0 per cent. starch solutions and amylase concentrations of 0·005-0·05 per cent. These workers, and also M. Schneider,[14] showed that with a constant value for the substrate/enzyme ratio, but at different dilutions, the reaction constant and the initial velocity first rise and then fall gradually with increasing dilution. Only the falling portion of this curve was examined by Schneider.

Vernon[15] has examined the influence of temperature on the reaction velocity of amylase action, and H. van Laer gives $K_{35}/K_{25} = 1 \cdot 93$; $K_{45}/K_{35} = 1 \cdot 31$, whilst the results of Lüers and Wasmund are given in Table XXXVIII.

TABLE XXXVIII

Temperature, °C.	K_{C+10}/K_C		
	Initial Value of k	*From Average Value of k*	*From Henry's Constant = 3·2*
20-30	2·046	1·899	1·96
30-40	1·675	1·688	1·65
40-50	1·294	1·506	1·43

Claus[53] has studied the action of β-amylase on 20 per cent. pastes of maize starch, which corresponds to the concentration often used in industry, at *p*H 5·5 and at temperatures between 48·5 and 68·5° C. The conversion to maltose, up to about 18 per cent. conversion, was found to be a zero order reaction. Above 43° C. the enzyme is inactivated exponentially by temperature. Claus gives the determination of the intrinsic activities of the enzyme and of its temperature inactivation indices by mathematical treatment of the curves.

4. *The Reasons for Deviations.*—As several workers,[4, 8] have found the velocity of hydrolysis of the intermediate products does not deviate much from that of the final products this cannot be the reason for the deviation from the unimolecular reaction. H. van Laer considers that the kinetics of amylolysis depends essentially on the 'diastatic loading' of the starch and thus depends upon the radio *substrate/enzyme*, but in view of the work already discussed this opinion does not clarify the situation.

The rectilinear form of the *time/change* curve up to R_M 40 is interesting. Here, in contrast to the demands of the law of

mass action, there is an apparent independence of the reaction on the amount of material already hydrolysed.

Duclaux[16] considers this to be specific for amylase and that the form of the second portion of the curve is due to the reaction products. E. F. Armstrong[17] assumed the formation of a system between the ferment and the substrate which is subject to the customary equilibrium, i.e. depends upon the amount of ferment, substrate and water (see also H. T. Brown[18, 19]).

H. T. Brown and T. A. Glendinning[4] pointed out that at the beginning of the action the amount of enzyme relative to the amount of starch is small, and as long as this excess of substrate remains unhydrolysed the ratio *starch/starch-enzyme complex* will be large. As long as this is so the starch enzyme complex will remain nearly constant in amount and equal amounts of starch will be hydrolysed in equal time, thus accounting for the rectilinear portion of the curve. Subsequently, when the starch concentration has been greatly reduced the amount of combination, and consequent hydrolysis, will follow more closely the law of mass action. If the initial concentration of amylase is large the straight line portion of the curve should change from the start and result in a reaction of the second order accompanied by a change in the value of k.

Lüers and Wasmund have applied Fodor's method[20] for examining polypeptide fission to amylolysis. They assume that the starch is adsorbed on to the surface of the amylase, the amount adsorbed being conditioned by the speed of diffusion of the starch and hydrolytic products to and from the surface of the amylase and also by the ratio—*surface/substrate concentration*. If the amylase concentration is small relative to the substrate concentration the adsorption, following Freundlichs' adsorption rule, is given by

$$\frac{y}{m} = Ac^{\frac{1}{n}}$$

where m is the amount of enzyme, y the amount of starch adsorbed, $1/n$ is a true fraction, A is a constant and c the equilibrium concentration of the starch in the liquid.

It is now assumed that reaction occurs only in the adsorbed layer and further that this reaction in the adsorbed layer is unimolecular, i.e. the rate of reaction is proportional to the concentration in the adsorbed layer, i.e. $\frac{dx}{dt} = k'y$.

This assumption gives

$$\frac{dx}{dt} = Ak'(a-x)^{\frac{1}{n}} = k(a-x)^{\frac{1}{n}}$$

where a is the initial concentration in solution and x the amount hydrolysed in time t and $k = Ak'$. Let $a = 1$, i.e. express x as a fraction of a then

$$k\int dt = \int \frac{1}{(1-x)^{\frac{1}{n}}} dx.$$

which gives

$$kt = \frac{n}{n-1}\left[1 - (1-x)^{\frac{n-1}{n}}\right].$$

Lüers and Wasmund obtained equations which agreed with their practical results very well, especially when the value of 4 was substituted for n which gives

$$k_4 = \frac{4}{3t}\left[1 - (1-x)^{\frac{3}{4}}\right].$$

For values of $n = 2$ and 3 the following equations are obtained :

$$n = 2 : k_2 = \frac{2}{t}\left[1 - (1-x)^{\frac{1}{2}}\right]$$

$$n = 3 : k_3 = \frac{3}{2t}\left[1 - (1-x)^{\frac{2}{3}}\right].$$

This conception of Lüers and Wasmund demands that the speed of reaction should increase with increasing number, because on account of the increasing dilution by decrease of the substrate concentration a progressively greater proportion of the starch is adsorbed and split on the surface of the amylase and, as stated above, the expression they derive agrees very well with their practical results.

L. Michaelis and M. Menten,[21] from their work on saccharose inversion, consider that the affinity between the enzyme and substrate determines the amount of combination between them. If the products of fission also had some affinity for the enzyme a competition would take place between starch and the dextrins and fission products of varying molecular weights for the amylase. This would retard the hydrolysis. M. Somogyi[22] supports this view and considers that the polysaccharides with the highest molecular weights have the greatest affinity.

S. Schwimmer,[59] after using α-malt amylase on amylose, amylopectin and glycogen, concluded that for all substrates having molecules of chain length of more than 10 glucose units the affinity constant K (see below) is invariant within experimental limits. The end products of the reaction, he thinks, inhibit non-competitively whereas incompletely hydrolysed products compete with the starch for the enzyme.

L. Michaelis and M. Menten[21] have calculated the dissociation constant K_m of the starch-amylase complex and have derived an expression analogous to that for the dissociation of an acid. The reciprocal of K_m is called the 'affinity constant'. K. Sjöberg and E. Eriksson[23] have found these values for amylose and amylopectin to be 2 and 2·5 respectively, which leads to the conclusion that both have the same molecular weight, a conclusion with which they disagree (see, however, Meyer and Press[42] above).

K. Myrbäck and N. O. Johansson[60] consider the rate of hydrolysis to be a function of the substrate concentration and define the 'affinity constant' as the reciprocal of the starch concentration expressed as moles. With maltose the rate of the hydrolysis is half the maximum rate.

(*b*) **Liquefaction.**—U. Olsson[24] has shown that the action of *a*-malt amylase on starch follows the form of a unimolecular reaction. A comparison of the reaction constants for the α- and β-amylases of malt shows that the liquefying action proceeds at a rate only 1·7 greater than the saccharifying action, and Olsson therefore considered that both reactions were catalysed by the same enzyme. He tried to prove this point by using poisons which he hoped would affect one reaction and not the other. The differences between the effects on the two reactions were so small that he considered his view confirmed.

G. Yamagishi[54] prepared *rice* diastase which appears to contain a liquefying, a dextrinising and a saccharifying enzyme. The reaction of the saccharifying enzyme on starch followed a unimolecular course up to about R_M 50 but a constant value for the first order reaction was not obtained with the dextrinogenic enzyme. The liquefying enzyme acted very speedily at the outset and then slowed down. After 1-2 days liquefaction was almost complete with an R_M value of only 9, and the blue colour with iodine persisted.

K. Myrbäck[58] found retrogradation to have no effect on the course of the hydrolysis and considers there is a connection between the degree of branching of the molecular chains of the substrate and the course of the reaction owing to the greater affinity of the enzymes for long chains than for short ones.

Pancreatic Amylase

It is interesting to note that several workers[25–27] have found that the relationship between the saccharifying and liquefying actions of pancreatic amylase is not altered by purification. They are, however, influenced in different degrees by various external factors and therefore their relative speeds differ with variation of enzyme concentration,[28] for example:

Concentration of pancreatic amylase	1	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{32}$
Ratio $K_{liquefaction}/K_{saccharification}$	34·3	45·5	45·6	62·9	47·0

By following the reducing power V. Henry[6] and Ch. Philoche[5] showed that the course of hydrolysis followed a unimolecular reaction in the first stage, and this has been confirmed by H. C. Sherman and J. C. Baker[40] for soluble starch and β-amylose up to R_M 40 and 50, respectively. The speed of reaction is definitely slower for amylopectin than amylose. R. Willstätter, E. Waldschmidt-Leitz and A. R. F. Hesse[29] obtained a hydrolysis limit corresponding to R_M 75, using a glycerine extract of swine pancreas, and the reaction was unimolecular up to 40 per cent. fission.

It is important to know the limits of enzyme concentration between which an exact proportionality of the concentration and the speed of reaction exists, for then the relative efficiencies of different preparations may be compared.

The reaction constants are inversely proportional to the substrate concentration in the case of both pancreatic and malt amylases but, according to Kendall and Sherman,[30] the speeds at the beginning are independent of the starch concentration between 1 and 4 per cent., and at lower concentrations they fall, but more slowly than the starch concentration. The speed is proportional to the enzyme concentration and, in the case of large amylase concentrations, G. Orestano[31] found a unimolecular course to prevail up to a degree of fission of 40-80 per cent. Pastes of native and of soluble starch were attacked equally fast.

R. B. Alfin and M. L. Caldwell[95] examined the action of highly purified pancreatic amylase on potato starch, Lintner soluble starch and maize amylose. In each case the extent of the hydrolysis depended on the concentration of amylase. Relatively very high concentrations of the amylase gave no evidence of a limit in the hydrolysis as is the case with β-amylase. The reaction curves show a rapid phase of reaction followed by a very slow

rate of change but the extent of hydrolysis attained was dependent, within wide limits, upon the concentration of the amylase. The slowing down of the reactions in this work did not appear to be due to any appreciable inactivation of the amylase or to interaction, or equilibrium, between amylase and substrate or products of hydrolysis, but to the replacement of the original substrate by products for which the amylase has lower affinities. Further work by F. Mindell, A. L. Agnew and M. L. Caldwell[56] confirmed these conclusions.

S. J. Pronin[32–33] noted high reaction rate during a short initial period after which the results can be fitted into a unimolecular equation. He found that in a reaction that ran for 2-3 hours this initial period was 15 minutes. After some 50 per cent. hydrolysis had occurred the reaction constant fell strongly. In the case of small enzyme concentrations and small reaction speeds there is an interval in which both a linear and a unimolecular hydrolysis may be concurrent. A. Janke and J. Holota[3] consider that with commercial bacterial and fungi preparations no constancy of reaction constants can be found, because the enzyme is attacking the starch molecule in a number of locations, thus producing a very complex type of reaction. K. Oshima,[41] however, finds that although different species of *Aspergillus* show variation in activity of the enzyme, the velocity of reaction follows a unimolecular formula.

The increased initial speed is apparently dependent on the colloidal properties of the substrate. G. Orestano[31] found the liquefaction of soluble starch to follow the unimolecular course up to 75 per cent. fission. The speed varied in an unproportionate manner with the quantity of enzyme but it probably shows itself proportional to the substrate concentration.

Salivary Amylase

Chittenden and, later, McGuigan[34] noted that starch is not entirely saccharified by salivary amylase, and McGuigan considers an inhibitor, which is not glucose or maltose, must be present. O. Hammarsten[35] finds that granules of different starches are attacked at different speeds, but on grinding the granules to break down their structure these differences are eliminated.[44]

When acting on solutions of Merck starch up to 1 per cent. strength for 30 minutes with a fixed amount of salivary amylase, the amount of sugar formed is the same irrespective of the ratio *substrate/enzyme* or of the concentration. When using the same amount of enzyme on 2-10 per cent. solutions, however, there

is a proportional decrease in the percentage of total starch saccharified.

H. von Euler and Svanberg[9] found, analogous to malt amylase, a unimolecular course for salivary amylase, the same saccharification limit and an almost equal reaction constant. C. L. Evans[44] and J. R. Broeze[36] found that the initial speed is proportional to the enzyme concentration when this is small. Assuming a 75 per cent. saccharification the reaction coefficients are proportional to the amount of enzyme in a given volume. J. A. Remesow[37] used a nephelometric method to follow the action of this amylase on rice starch and concluded it followed a monomolecular course.

K. Myrbäck[38] finds that in the presence of the nitrate ion the reaction constant is much lower than in the presence of the chloride ion. With regard to the temperature coefficient from 10-30° C. this has a value of 2, but from 30° C. upwards it falls rapidly, possible because of the increased dissociation of the sodium chloride-ptyalin compound with increase of temperature. As already discussed earlier the presence of sodium chloride is essential to the activity of this amylase and a loose combination between the salt and amylase appears to take place.

With both salivary and pancreatic amylases the termination of the dextrinogenic stage of the hydrolysis is not marked by any obvious slackening in the rate of hydrolysis such as is observed in the case of α-malt amylase. C. S. Hanes[39] suggests that it is probably that the first two α-amylases have higher affinities for short-chain dextrins (in relation to their affinities for the more complex components of the substrate) than α-malt amylase. This might or might not be related to the presence of another component in the animal preparations. Such a conception would provide a basis for interpreting the general forms of the hydrolysis curves, the different but characteristic achroic R_M values, and the fact that for salivary and pancreatic α-amylases this value increases with increasing substrate concentration, but conclusive evidence is not available to support this conception. M. A. Swanson[37] found the action of salivary amylase on starch to be more random than its action on glycogen and also examined other amylases.

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Chapter 24

METHODS OF DETERMINING THE ACTIVITY OF AMYLASE PREPARATIONS

There does not appear to be any entirely satisfactory and rapid method for determining the activity of enzyme preparations used in the various industries although there are several of great indicative value. In the textile industry complications arise from the use of the different starches[25–26] which may be employed for different types or even the same type of work, and which may give somewhat different results in large-scale working. The methods of preparation of different sizes, etc., and the after-treatments, such as drying, to which they have been subjected, may tend to alter the starch present so that it is not strictly comparable with untreated starch. The presence of inorganic salts in certain dressings and starches may act as poisons for a particular enzyme preparation and thus lead to waste or faulty results when applied on a large scale. These considerations are of particular importance in the textile industry.

Even in certain industries where standard methods have been adopted, two classes of error may appear. H. R. Sallans and J. A. Anderson,[44] when investigating the official method of the American Society of Brewing Chemists for estimating the diastatic power of malt, point out that the first class of errors embraces those inherent in any particular empirical method, such as that just mentioned, and into the second class fall those due to the use of unofficial modifications and non-observance of certain standard procedures. In the first class the chief errors are those arising from the faulty preparation of solutions and the vexed question of the determination of reducing power (*vide infra*).

Variations in the starch used, in the temperatures of extraction of the malt, and the method of sampling malt, introduce errors of varying magnitude which have been determined by the above workers. As examples of the second class of errors, departure from the standard ratio of water to malt during the extraction and disregard of Kjeldahl's law of diastatic conversion may be mentioned. Centrifugal separation of the malt extract from the insoluble matter was found to give similar results to the filtering

recommended in the above official method, but if the liquid was separated by settling and decantation, as in the standard method of the Institute of Brewing, lower results were obtained. The optimal conditions of activation, *p*H value, time and temperature for the extraction of the total β-amylase in barley and malt have been investigated by S. R. Snider[107] who uses a mixture of papain and cysteine to free the β-amylase. He claims that this mixture allows as much β-amylase to be extracted in 3 hours as can be extracted using papain alone in 24 hours and that the new method is more accurate. After correcting for malting losses the green malts showed considerably more diastatic power than the corresponding barleys, but on destruction of the α-amylase in these malt extracts the diastatic powers of barley and malts agreed fairly closely.

T. Chrzaszcz[51] has repeatedly stressed that the activities of diastases should be considered separately according to whether they are liquefying, dextrinising or saccharifying in action. Windisch[52] considers that the liquefying and saccharifying actions do not appear simultaneously.

Anderson and Sallans[104] have modified the ferricyanide method (*vide infra*) but A. D. Dickson[105] finds that even this modification gives results which can be grouped into a high and a low class, within each of which there is very good agreement, but that there is no consistent association of low duplicate errors with either group. This worker, in a collaborative study of three malts examined by nine collaborators on each of four different days, each worker using the same soluble starch, concludes that it is necessary for each laboratory to examine its own sources of error. The results of his study have led the Malt Analysis Standardisation Committee (U.S.A.) to recommend the ferricyanide method, as well as that of the American Society of Brewing Chemists which is similar to that of the Institute of Brewing, for the determination of diastatic power. A further collaborative study of the two methods[108] has shown almost identical average values at the four levels of activity and variations between two laboratories were similar. It also verified the conversion factor for ferricyanide to degrees Lintner suggested by Anderson and Sallans. This factor is 23 when the American Society of Brewing Chemists' method is employed. A third collaborative study[109] showed the two methods to give values of the same magnitude. Dickson, therefore, recommends that the ferricyanide method be adopted tentatively as an alternative to Fehling's method and that Rask's[110] suggestion that values for diastatic power be reported as degrees Lintner can be dropped.

For description of both of the revised methods the reader is referred elsewhere.[111]

The Units of Amylase Activity.—The oldest unit still in use is that due to Lintner,[13] who expresses the power of a diastase as 100 when 0·3 ml. of a solution containing 0·1 gm. diastase in 250 ml. water produces sufficient sugar to reduce 5 ml. of Fehling's solution when acting on 10 ml. of a 2 per cent. starch solution at ordinary temperatures for 1 hour. The reaction constant can be found from this value in the following way[74]: 200 mgm. of starch have been used, and supposing 75 per cent. fission has occurred then $a = 150$ mgm., and since 5 ml. of Fehling's solution represents 0·03682 maltose, $x = 36 \cdot 82$ mg., $a - x = 113 \cdot 18$ mg. $\times k$, the reaction constant is 0·00205.

Osborne[54] uses Lintner units, and Effront[55] bases his estimations of reactive power on the saccharification which occurs under certain conditions at 60° C. This is not too happy a choice, as at 60° C. the enzyme is already affected by the heat. Consideration of the limiting value of saccharification at concentrations up to 40 per cent., i.e. within the range at which the proportionality law holds in certain limits of enzyme and substrate concentration shows that the equation for the monomolecular reactions yields values of k which are constant for practical purposes. In view of this the amylase activity has been measured by several workers by the value of the reaction constant.

Sherman and co-workers[8, 57] saccharify 2 gm. of soluble starch for 30 minutes at 40° C., and estimate the Cu_2O which is produced from an oxidising copper solution. A connection between the amount of Cu_2O formed and the reaction constant is readily obtained, and to convert this constant into the 'new scale' units it is divided by the quantity of enzyme used, e.g. if 0·0312 gm. of enzyme are employed, the amount of Cu_2O precipitated is 100 mgm. This is equivalent to 82 mgm. of maltose from which $k = 31 \cdot 2$ (*vide supra*), and therefore $31 \cdot 2 / 0 \cdot 0312 = 1{,}000$ units.

H. v. Euler and O. Svanberg[58] define the saccharifying power by the equation $Sf = k \times g$ (maltose)/(gm. preparation) in which k is the monomolecular constant for saccharification, and g (maltose) is the number of grammes of maltose which can be obtained in a reaction measured by k, and (gm. preparation) is the amount of enzyme present. This relation characterises amylase preparations in 0·72 to 2 per cent. starch solutions and such preparations which give reaction constants of 0·004 and 0·08 at 37° C. in the presence of suitable neutral salts; 1,000 Lintner units correspond to about 26Sf and 1,000 units of the 'new scale' equal 38·5Sf.

Willstätter[59] takes as an amylase unit one hundred times the amount of enzyme, which gives a value of $k = 0 \cdot 01$, therefore the constant expresses at the same time the number of the amylase units in the test. The degree of purity of a preparation is expressed by the *amylase value* or the number of amylase units in 1 gm. of the dry preparation. The Euler-Svanberg unit refers to 1 gm. of enzyme preparation, and to convert Sf into the 'amylase value' it is multiplied by 0·05333, i.e. amylase value $= 0 \cdot 05333Sf$. Euler and Svanberg propose 37° C. as a normal working temperature for comparing amylases, and it has been found that $k_{30}/k_{20} = 2 \cdot 0$ and $k_{40}/k_{37} = 1 \cdot 2$.

R. M. Sandstedt and co-workers[95] suggest that the α-amylase present in malt be expressed as the number of grammes of soluble starch that, in the presence of excess β-amylase, are dextrinised by 1 gm. of the enzyme in 1 hour at 30° C.

Lintner soluble starch is generally used as substrate in the determination of the amylase units, but it is not an enzymatically uniform substance, as mentioned below. Different amylases affect it in different ways, so that it may be used to compare the activities of the same type of amylase but not of different types of amylase preparations. H. v. Euler and K. Josephson[60] find that Lintner starch gave more maltose at the end of the reaction than did Zulkowsky's soluble starch. The constants are not affected, however, if values within the first 40 per cent. of fissions are employed.

J. Blom, A. Bak and B. Braae[61] use viscosity measurements to determine activity and compute this quality by determining the time in seconds (t) in which v gm. of amylase reduces the viscosity of a starch paste to that of a reference solution consisting of 50 per cent. saccharose in water. The unit of activity (V) is given by the equation $V = 1{,}000/t.v$. If t is the time in seconds for the iodine colour to match that of a comparison solution and v is as above, the same expression holds true. Other units are mentioned below, principally the 'liquefon'.

Methods of estimating the activity of an enzyme-preparation may be broadly classed under five main headings, and from these methods may be selected one that will suit the requirements of any particular problem. The five classes are as follows:

I. Viscosity methods.[1]

II. Methods involving the determination of reducing power.

III. Iodometric methods, i.e. noting the amount of enzyme required to destroy the iodine reaction.

IV. Direct determinations on sized cloth. 3-4

V. Miscellaneous methods.

1. Viscosity Methods.—T. Chrzaszcz[62] and J. Janicki[63] have examined the methods in use for determining the liquefying power of amylases, and give 15 methods, together with 18 proposed modifications of these methods. These methods are divided into 3 groups by these workers. In the first group come those in which the liquefaction of a starch paste to which the enzyme has been added either before,[55, 62–67] or after the starch has been pasted as in Lüers' method,[68] or those of A. Pollak,[69] U. Olsson,[70] S. A. Waksman,[19] and others.[64, 72] The second group embraces those methods in which the decrease in viscosity of solutions of soluble starch,[64] such as those of L. Fletcher and J. B. Westwood,[39] A. J. Hall,[3] and others[2] (*vide infra*). In group 3 come those methods such as those depending on iodine coloration or sugar determination. S. Jozsa and H. C. Gore[2] have followed the changes in viscosity of a remarkably uniform starch paste as a means of determining the liquefying power of the diastase used. A. Janke and J. Holota[94] have determined the liquefying power of commercial α-amylase preparations (Biolase and Superclastase) on 5 per cent. potato-starch pastes at various pH values and different temperatures and use the Höppler viscometer to follow the reactions. L. Fletcher and J. B. Westwood[39] suggest that erroneous values for the liquefying power of the enzyme are obtained if the amount of starch liquefied is assumed to be proportional to this power unless a restricted experimental range is used, and even then the results are not entirely satisfactory.

J. J. Willaman, E. W. Clark, and O. B. Hager[27] greatly modify this method, but inaccuracies still enter into the result due to the application to any diastase solution of certain empirical constants for a given preparation. Their method also does not take into consideration the rate-curves of different samples, especially at the point where little starch is left; and doubling the concentration of the active agent does not double the liquefying power, as the latter, by this method, represents an average value only for the rate of conversion and is not proportional to the actual enzyme-content.

K. Mayer[99] has introduced, as a substrate for the determination of the liquefying power of enzymes, starch which has been treated with iodine in amount insufficient to give a blue colour. To 100 ml. of a 1 per cent. starch paste is added 0·65 ml. of N/100 iodine and the resultant product is resistant to starch saccharifying

zymes but the action of liquefying enzymes and the iodine coloration are unaffected.

S. Jozsa and W. R. Johnson[28] have introduced a new unit of enzyme-activity which they term the 'liquefon', and by its use they claim to be able to calculate accurately the diastatic activity of enzyme preparations from the measurement of the viscosity changes in a starch paste.

They define the 'liquefon' as the amount of starch-liquefying enzyme which will convert a standard starch paste at the rate of 25 mg. of dry starch per minute at zero time under the given experimental conditions. This unit has been fully discussed by these workers[29] elsewhere.

A starch paste (2,000 ml.) is made containing 4·211 gm. of dry starch per 100 ml. paste, stirred with a high-speed stirrer for about 1½ minutes while hot, cooled to 20° C., buffered to *p*H value 5-5·2 with 50 ml. of Walpole's acetate buffer, made up to the mark with distilled water, and then again stirred 30-45 seconds to obtain a uniform paste. A test portion of this paste is mixed with 10 per cent. its weight of a solution of sodium chloride containing 25 mg. salt per 100 ml., and stirred for 1 minute in the high-speed stirrer. The time of outflow from a water-jacketed pipette at 21° C. is determined and should correspond within two seconds to the time of outflow of an 81 per cent. solution of glycerol (sp. gr. 1·2138 at 20-21° C.). A pipette is chosen with a delivery-time of 55-57 seconds for 100 ml. of water, the time of delivery of the same amount of the specified glycerol solution being between 165-190 seconds at 21° C. If the delivery-time of the starch paste is not within two seconds of that of the glycerol solution, the stirring is continued until it is, and the length of time of stirring is accurately determined.

It should be noted that a fresh starch paste should be made up each day, and that the stirring-jars should be of such dimensions that the whole of the paste is well-stirred.

A 150 gm. sample of the starch paste prepared as above is cooled to 19·5° C. and 15 ml. of the enzyme solution (see below) or sodium chloride solution (for blank tests) stirred in, the correct time of stirring being determined by running one or two blanks, as described above. After stirring, the temperature should be 21° C. ± 0·2° C., at which the paste is maintained for 59 minutes; then it is sucked into the pipette and the time of outflow of 100 ml. determined. The viscosity determination is begun before the end of 1 hour to correct for the liquefaction occurring during the determination. To check the stability of the paste, another blank should be run on 150 gm. of paste that has stood for 1

hour at 21° C. This value should not deviate by more than 3-4 per cent. from the original values.

From the outflow-time of a given mixture, Jozsa and Johnson obtain the amount of starch liquefied by use of the formula: $S = 12 \cdot 9\ P - 0 \cdot 065\ P^2 + 0 \cdot 0025\ P^3$, where S is the number of mg. starch liquefied, and P is the percentage decrease in time of outflow between the blank and the sample. The enzyme content or activity of the solution is derived from the amount of starch liquefied by means of the empirical formula: $\text{Log}_{10}L = 0 \cdot 000565\ (S - 1{,}078)$, where L is the number of 'liquefons' in 10 ml. of infusion, and S = mg. of starch liquefied in 1 hour. From the concentration of the infusion the number of L in 1 gm. of the preparation is calculated; it is an exact measure of the α-amylase content and of the liquefying power at zero time. The following example taken from the original paper is given in illustration:

	Outflow-time in Seconds
Paste stirred with water for 60 secs.	181·5
Above paste after additional stirring (trial)	164·5
Another sample stirred with water for 65 secs.	171·2
Glycerol solution	170·0
Initial outflow-time	171·2
Final outflow-time after enzyme-action is complete	56·5
Range	114·7
Outflow-time after 1 hour at 21° C. using sample (10 mg./10 ml.)	91·8
Decrease in outflow-time (171·2-91·8)	79·4
Percentage decrease 7940 ÷ 114·7	69·2
Milligrams of starch liquefied (by formula)	1408·4

Now $\text{Log}_{10}L = 0 \cdot 000565\ (S - 1{,}078) = 0 \cdot 1870$; $L = 1 \cdot 538$ and as 10 mg. of enzyme sample were used, 'liquefons' per gm. = 154. As the 'liquefon' is the amount of enzyme that will convert 25 mg. of dry starch per minute at zero time, the liquefying power of this sample at zero time is 3,850 mg. of dry starch per minute.

To prepare the enzyme solution, a suitable quantity of the preparation (2-15 gm.) is weighed into a 1,000 ml. flask, 25 gm. of sodium chloride added, and the contents made up to the mark. After standing 1 hour with occasional shaking the infusion is filtered, the first 100 ml. of the filtrate being rejected; 100 ml. of the remainder are then transferred to a 1,000 ml. flask and made up to the mark.

To obtain good results the liquefaction in 1 hour should be between 50 and 90 per cent., and with materials of 1-3 'liquefons' per gm., an infusion of 10 gm. of enzyme material in 10 ml. is required to bring about liquefaction with the above range. Strong preparations of 1,000-3,000 'liquefons' per gm. require only 1 mg. per 10 ml. for this purpose.

A simple procedure and apparatus for use in factory routine-testing is described by A. J. Hall,[3] in which a 3 per cent. starch paste is treated with a definite quantity of a 0·4 per cent. solution of a diastase preparation at 21-22° C. for 40 minutes, and the viscosity determined. U. Olsson[70] measured the decrease in viscosity by measuring the rate of rise of a hollow glass sphere through the reaction mixture but considers the Höppler viscometer preferable to any other instrument.

2. Methods Involving Determination of Reducing Power.—Methods that are based on the amount of sugar formed by the completed diastatic action of malt on starch, e.g. in the method of E. F. Harrison and D. Gair,[4] are not so accurate as could be desired, because the amount of liquefaction is not related to the amount of sugar formed, two distinct processes entering into the reaction—liquefaction by one enzyme and saccharification by another. Also, in the case of some preparations the enzymes have different sensitivities to heat and other external agents and are affected to different extents. J. Kjeldahl,[5] however, has shown that if malt extract is allowed to act upon starch at 57-59° C. until about 40 per cent. of the starch has been converted, the reducing sugar formed is directly proportional to the diastatic power. Practically all the methods employing estimation of the amount of reducing sugar take this observation of J. Kjeldahl into account.

The reducing power (R_M) is expressed as the percentage of the reducing power that would be shown under the same conditions if the substance could be completely transformed into anhydrous maltose ($[\alpha]_D = 137 \cdot 9°$). One gramme of starch would yield theoretically 1·055 gm. maltose. When maltose is the sole reducing product formed, as happens with the saccharogenic amylase of malt, this convention is devoid of ambiguity and provides an index of the amount of transformation of starch to maltose that has occurred. With most amylases, however, particularly in the early stages of the action, this is by no means the case and the significance of the conventionally expressed reduction value may obviously be different in such cases. Thus an R_M of 36 could indicate either 36 per cent. of the substrate has been converted to maltose, but it could equally mean that the whole of the substrate had been converted into reducing dextrin having a chain-length of 6 glucose units. Thus R_M gives an indication of the number of linkages hydrolysed but not of their relative positions in the original chain structure nor of the nature of the products containing the reducing groups. J. Blom, Bak, and Braae[61] suggest that the method of determination could be

indicated by suffixing to the symbol R_M the initial of the worker whose method is employed, e.g. malto-dextrin, R_{MB} (or RMB) = 43 if the Bertrand method is used, or R_{MW} (or RMW) = 43 if the Willstätter method is employed. In this case the malto-dextrin would have 43 per cent. of the reducing power of anhydrous maltose. They suggest a similar means to denote the degree of degradation of starch and the method employed. The amount of maltose stoichometrically equivalent to the starch destroyed is expressed as a percentage of the amount of maltose stoichometrically equivalent to the whole of the starch employed, and this figure represents the degree of degradation of the starch. This figure would, of course, be affected if some other disaccharide residue is present in starch, but convincing evidence of such an eventuality has yet to be produced.

C. J. Lintner[13] was the first to introduce the use of soluble starch for determining diastatic activity. Starch treated with 7·5 per cent. hydrochloric acid for 7 days at ordinary temperature, or 3 days at 40° C., and then washed free from acid, gives a solution which remains clear for days, and is devoid of the usual paste-forming properties of the untreated starch. The amount of reducing compounds present is practically negligible. Lintner adds varying amounts of diastatic solution to 10 tubes containing the same amount of starch, and after 1 hour 5 ml. of Fehling's solution is added to each, and they are heated on a water-bath. The tube in which all the copper has just been reduced is noted.

Two considerations affect the selection of a suitable method, speed and accuracy, and dissatisfaction is often expressed with methods for determining the reducing sugars left after the diastatic action. The high degree of accuracy claimed for some of the methods hardly appears warranted in the final results when the difference in the time of manipulation is considered.

The method adopted by the Institute of Brewing is that of Lane and Eynon[34] which is quite satisfactory except that inexperienced workers may obtain discrepant results, but this probably applies to other methods also. S. Laufer, E. Schwarz, and L. Laufer[46] consider that the original Hagedorn and Jensen method gives better results than the official method of the American Society of Brewing Chemists.

The amount of reducing sugar formed may be determined volumetrically or by gravimetric methods,[6–7] preferably by that of H. C. Sherman and his co-workers.[8] J. T. Flohill,[9] for example, allows the enzyme to act on a 2 per cent. solution of soluble starch at 20° C. for 1 hour, after which caustic soda is added to destroy the enzyme, the solution diluted and boiled with Fehling's

solution. The amount of reduced copper is determined by adding potassium iodide solution plus sulphuric acid and titrating with sodium thiosulphate. The rate at which Fehling's solution or other alkaline copper solution is used up is dependent on the nature of the reducing substances and on the method employed. If the solution contains sugar alone concordant results are obtained from both alkaline copper reagents and iodometric methods, but the greater the reducing power attributable to any dextrins present the more the results obtained by the two types of method diverge. When dextrins are present, therefore, Bertrand's[71] or Fehling's methods are inferior to iodometric methods.[73]

Sherman and his co-workers,[8] and C. A. Brown[30] criticise adversely the classical Lintner[13] method. If *p*H control is available the Sykes and Mitchell[31] method is probably the most precise of the modified Lintner methods for estimating the saccharifying power by malt diastase, but owing to filtration difficulties it is too long for control purposes. Blish and Sandstedt[32] consider that the usual methods for determining the reduced copper in Fehling's solution fail to give concordant results when used by different workers, possibly owing to back-oxidation of the reduced copper, as noted by Hanes.[33] Of other methods based on the reduction of alkaline copper solutions, those of W. Engelhardt and M. Gertschuk[77] and C. Wirth[78] should be mentioned, while modified Lintner methods for determining the diastatic activity of malts have been described by W. Salač[84] and others.[85–89]

Timing errors are reduced to a minimum by the method of Sherman and co-workers, in which the starch is added to the malt and digested at 40° C. W. Syniewski[101] considers that when Lintner's soluble starch is used for the determination of diastatic activity the results are low, as he found that the first small quantity of diastase added caused no saccharification. With fresh starch solution the error is small but becomes considerable if the solution is stored for a few days before use.

C. T. Bennett and F. C. L. Bateman[102] are further workers who stress that the variability of the soluble starch causes inconsistencies in assessing the diastatic power of malt and malt extracts. They also recommend that attention should be paid to the freedom of the water and the laboratory atmosphere from ammonia and nitrites.

Lane and Eynon's[34] method is one of the standard methods used by workers in England, but H. C. Gore and H. K. Steele[38] consider that it is not precise enough to be used with highly active malts.

A volumetric method has also been devised[10] which utilises the conversion of maltose to maltobionic acid by means of iodine in the presence of caustic soda. The malt extract is allowed to act upon a 2 per cent. solution of soluble starch for 30 minutes at 20° C.; the action is stopped by the addition of caustic soda, and the maltose present oxidised to maltobionic acid by the addition of standard iodine solution, the mixture then being back-titrated with standard sodium thiosulphate, the excess standard iodine acting as the indicator. W. Windisch and P. Kolbach[11] make a correction in this method for the amount of iodine absorbed by the diastatic solution and their method is widely used on the Continent. In a further paper they[12] ascribe the varying results obtained by this method to variations in the *p*H value and in the state of dispersion of the starch. The hypoiodite method is considered inadequate for highly active malts and has been somewhat adversely criticised by Kline and Acree[35–36]. Samec and Blinc[74] consider that a stoichometric relationship is shown by the iodometric method of Willstätter and Schudel,[75] or that of F. Auerbach and E. Bodländer,[76] and that they may be used with every confidence.

Hagedorn and Jensen's[37] method for determining sugar in blood has been modified by Hanes (*v.s.*) for the exact titrimetric estimation of the reducing substances as maltose, formed by malt diastase, and for accuracy and convenience it leaves little to be desired. A further modification of the method by Blish and Sandstedt for determining reducing substances formed by the action of diastase on flour suspensions has been found to give excellent results. Larger amounts of maltose can be determined by this method than by Hanes' method; H. C. Gore and H. K. Steele[38] have used the reagents specified in the former for determining Lintner values and find it both rapid and accurate.

Twenty-five grammes of finely ground malt are digested with 500 ml. of water at room-temperature for 1½ hours with occasional shaking, after which it is filtered, the first 50 ml. of filtrate being rejected. For syrups and liquid extracts multiples or fractions of the 5 per cent. infusion are prepared; 25 ml. of the filtered infusion are diluted to 250 ml. and 10 ml. of this dilute infusion transferred to a 200 ml. volumetric flask and kept at 20° C. At a noted time 100 ml. of soluble-starch solution, containing 2·2 gm. Lintner's soluble starch and buffered with Walpole's acetate buffer are delivered, the time being counted from the time the first drop enters the infusion. The liquid is kept rigidly at 20° C. for 30 minutes from the time of starting the clock, after which 20 ml. of a 0·4 N caustic soda solution are added, the first

portions of the alkali being mixed with the solution as quickly as possible to stop the diastatic action. The alkali is neutralised with 20 ml. of 0·4 N hydrochloric acid and the volume made up to 200 ml., and 25 ml. of this solution are thoroughly mixed with 50 ml. of alkaline ferricyanide reagent, made by making 16·5 gm. potassium ferricyanide and 22 gm. of anhydrous sodium carbonate up to 1 litre with distilled water. The mixture is heated for exactly 15 minutes on a boiling water-bath, keeping the liquid in the flask below the level of the boiling water. After cooling to room-temperature, 125 ml. of an acetic acid reagent (200 ml. glacial acetic acid, 70 gm. potassium chloride, 20 gm. crystalline zinc sulphate, made up to 1 litre) are added, and then 5 ml. of a 50 per cent. potassium iodide solution; the iodine liberated is titrated with 0·05 N sodium thiosulphate solution.

To obtain a blank experiment, 10 ml. of the infusion are mixed with 20 ml. of 0·4 N caustic solution, neutralised with 20 ml. of 0·4 N hydrochloric acid, and 100 ml. of the starch solution added. The solution is then made up to 200 ml. with distilled water and 25 ml. of this are mixed with 50 ml. of the alkaline ferricyanide reagent, the procedure from this point being the same as for the sample.

The degrees Lintner (L) of the sample are given by the formula $L = 8 \cdot 092\ F$, where F is the net volume of the ferricyanide reagent consumed.

F. W. Norris and W. A. Carter[47] titrate the sugar solution against alkaline ferricyanide, using methylene blue as indicator, thereby eliminating the back-titration necessary in most ferricyanide methods, and obtain results which are comparable with those obtained with Lane and Eynon's method.

The amount of ferrocyanide formed by the action of the sugar on the ferricyanide is determined by F. C. Hildebrand and B. A. McClellan[48] by titrating with ceric sulphate solution, using 'Setopaline C' as an internal redox indicator, the results agreeing well with those obtained by the Blish and Sandstedt method. V. Jerschov[49] proposes to use either chemical or polarimetric methods, but his directions in the first stages are somewhat novel.

C. F. Silbernagel[15] considers that refined potato starch is a much better standard reagent than the soluble starch made from it, and he therefore proposes to use it for estimating both the liquefying and saccharifying powers of a malt diastase.

In the method of T. M. Back, W. H. Stark and R. Scalf[112] for determining the *limit dextrinase activity* of a preparation the substrate is a standard limit-dextrin solution and the activity is expressed as the mg. of fermentable sugar produced from this

standard solution by 1 gm. of the enzyme in 1 hour at 30° C. The method can also be used to determine the effect of variables, such as temperature, *p*H value and reaction time on limit dextrinase activity.

3. Iodometric Methods.—The degradation of starch when acted upon by dextrinogenic amylase may be followed by observation of the iodine reaction taking place. The quantity of enzyme necessary to reach the stage at which the blue colour turns to red (the erythro point) or alternatively the point at which the colour entirely disappears (the achroic point) in a constant time[16] is determined, or, as in the method of J. Blom, A. Bak, and B. Braae,[61] the time elapsing between the addition of the enzyme and the moment of reaching the erythro or achro points.

When using malt extract the iodine method, as mentioned below, is unreliable. When it is realised that the amounts of dextrinogenic and saccharogenic amylases may vary over wide limits for various malt extract preparations and that the action of each is influenced to a different extent by certain unlike factors the inherent sources of error in this method of assessing activity will be seen. The action of mixtures of α- and β-amylases is discussed in Chapter 22.

Sherman, Kendall, and Clark[8] have modified the method of J. Wohlgemuth,[16] in which the enzyme preparation acts on a given amount of soluble starch in solution for 30 minutes at 40° C., and the amount of enzyme necessary to effect the disappearance of the blue coloration with iodine is noted. The results are expressed as the number of ml. of standard solution digested by 1 ml. of enzyme solution under these conditions. Dymond,[22] D. L. Davoll,[23] V. Dorfman[97] and Scheermesser[24] have employed variations of Wohlgemuth's method, but Davoll considers it unreliable owing to variations in the ratio of dextrin to maltose. When pure β-amylase hydrolyses amylose the iodine coloration remains blue until over 90 per cent. maltose has been formed, when the colour turns violet and then red.[90, 91] Giri[92] finds that sweet-potato amylase behaves in this way, and concludes that it is practically pure β-amylase. This worker[93] finds that the iodine colour reaction with different starches varies markedly with the kind of starch hydrolysed and the concentration of iodine used. Samec shows that the colour tone-intensity changes during amylolysis. With β-amylase, however, although the blue colour is retained to beyond the 90 per cent. saccharification stage the intensity of the colour with iodine diminishes as the hydrolysis proceeds, and smaller quantities of iodine are required to attain the maximum coloration of the solution. Samec and Mayer[79, 80]

have evolved a method in which the maximum intensity of blue colour obtainable at each stage of hydrolysis is compared with the initial intensity or with a standard starch iodide solution. Every stage of the hydrolysis is then represented by characteristic numbers which can be plotted in a similar manner to the values obtained from measurements of optical rotation or reduction values.

T. Sabalitschka and R. Weidlich[53] follow the reaction to the erythro point by use of comparison solutions of methylene blue and safranine.

R. M. Sandstedt, E. Kneen and M. J. Blish[95] point out that in the Wohlgemuth method of measuring α-amylase activity the presence of β-amylase causes some doubt as to the true end-point, owing to the variable effect and amount of this substance in malt extract. In combinations where the saccharogenic power of the β-amylase is more than twice that of the α-component, a condition generally characteristic of malts, the saccharogenic activity of a mixture of the two amylases is the sum of the individual activities when the components act separately. They overcome this by the addition of β-amylase in such an amount that its further addition does not further increase the rate of dextrinisation. When this is done they find that there is a linear relationship between the α-amylase content and the time of dextrinisation. In a further paper[106] they give a rapid method for measuring the β-amylase activity of barley malts using, however, a ferricyanide method for estimating the maltose formed. A method incorporating these observations has been evolved by L. E. Ehrnst, G. J. Yakish and W. Olson.[96]

W. A. Johnson[14] digests potato-starch pastes with increasing proportions of a solution of the diastatic preparation for 10 minutes at 40° C., and notes the amount required to be added to obtain no iodine reaction at the end of the period. He finds that the results closely agree with those obtained by determining the amount of sugars formed. The absorption spectrum of starch-iodide in various stages of hydrolysis has been followed by Hanes and Cattle[56] by means of the spectrophotometer (see Chapter 10). The differentiation of starches and of some amylases by the iodine reaction has already been discussed.

4. Direct Determination on Textiles.—From the point of view of factory control it is preferable to have some method of determining the activity of a desizing enzyme on the cloth upon which it is subsequently to be used. Such a method has been elaborated by Clibbens and Geake,[17] who determine the sizing in a piece of cloth of known weight by treating it in a desizing

enzyme solution, wringing it out by hand twelve times in a stream of running water, and then drying to a constant weight. Such a procedure may lead to variable results owing to the wringing being done by hand, but it might be possible to standardise a procedure whereby the fabric was passed through a squeezing roller set to a known expression, say 80 per cent., then suspending the fabric in running water for a determined time and repeating the whole process a definite number of times. They found that 3 per cent. of the total weight of cloth should be ascribed to the waxes and non-sizing agents and gums removed from the cellulose, and allowance must be made for this in determining the weight of size removed. This correction is applied to American, Egyptian, and South American cottons, but rayon fabrics do not require it. Using this method the comparative activity of several enzymes could be determined, the results giving an indication of the most advantageous preparation for technical use.

D. H. Powers[18] suggests a modification of this method in which 10 gm. of cloth, dried to a constant weight, are treated with 200 ml. of a 5 per cent. desizing solution for 30 minutes at 135° F. in a stout jar. A number of Monel metal balls are added and the whole shaken or rotated at frequent intervals. At the end of the 30 minutes the desizing solution is drained off, replaced by 200 ml. of a 0·5 per cent. soap solution, and the whole maintained at 160° F. for 15 minutes with constant agitation. The soap solution is replaced by an equal amount of water at the same temperature and the whole agitated for 15 minutes, when the cloth is drained and dried to a constant weight. The washing machine of the Society of Dyers and Colourists would be very suitable for this purpose, and the results used in the manner suggested above.

W. M. Scott[98] has evaluated the desizing efficiency of enzymes by two methods, one involving immersion, the other padding, which correspond, respectively, with the actual jig and padding methods used commercially.

5. Miscellaneous Methods.—S. A. Waksman[19] stains the starch in a 2 per cent. paste with Neutral Red, maintains the mixture at 40° C., and notes the time taken by the opaque solution to become clear.

A polarimetric method[20–21, 41] has been introduced by H. C. Gore, who determined the relationship between the reducing power and the decrease in polarisation before and after mutarotation was destroyed by the action of ammonia. The mixture of soluble starch and diastatic infusion must be held within the *p*H range of 4·5 to 5·5 to obtain correct results. During sacchari-

fication Kjeldahl's law of proportionality is followed, and the fall in polarisation observed is a little over 3° V. This author[40] later increased the magnitude to 11·3° V. by the use of a special type of soluble starch from which comparatively strong solutions could be prepared, and thus increased the accuracy of the method. The starch is prepared by treating it for 6 days with 1½ times its volume of 13 per cent. hydrochloric acid, after which it is well washed with distilled water, the last trace of acid neutralised with ammonia, and the product dried in warm air.

Another soluble starch has been suggested by S. R. Snider and D. A. Coleman,[42] but M. Rossatkevitsch[43] considers that soluble starches of different origin can give variations in the diastatic value which is obtained from the same malt, clear solutions giving higher values than opalescent solutions. They point out that the diastatic value decreases with the concentration of the starch, and that the optimum *p*H value for a given malt varies also with this concentration. All these points emphasise the necessity for adhering strictly to the requirements of standard methods, so that the results obtained by different workers may be comparable.

Rona and Eweyk[81] have used a *nephelometric* method for the examination of the hydrolysis of amylo-amylose with β-amylases, but the examination of α-amylases by this method cannot be carried out owing to the partial coagulation which takes place in the solution. J. A. Remasow[82] tests various amylases by this method, using a rice starch which has been solubilised by heating under pressure, and found that at concentrations up to 1 per cent. the Kleinman and Beer law holds for dilution, but for higher concentrations a correction must be applied to the results.

O. Wolff[100] has followed the course of the action of diastase on starch, using the Zeiss interferometer, which he considers is far more sensitive than the refractometer for this purpose. After a simple calibration with mixtures of sugar and starch solutions the readings obtained are claimed to be proportional to the amount of diastase present. Traces of copper, a well-known poison for diastase, in the water was found by D. Schenk[103] to reduce the apparent diastatic power of malt extracts to a half or even to one-third of their real value.

H. B. Sreerangachar[83] has used a *dilatometric* method to follow the hydrolysis of starch and its components by takadiastase. For soluble starch he found a linear relationship between the falling off of the dilatometric value, the amount of sugar formed and the change in optical rotation.

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